# BIOGENESIS OF SECRETORY GRANULES IN THE BOVINE ADRENAL MEDULLA

James Grant Pryde BSc. MPhil.

Thesis for the Degree of

Doctor of Philosophy

University of Edinburgh

1987

for

Irene

without whom

none of this would have been possible

....the experimenter doubtless forces nature to unveil herself by attacking her with all manner of questions; he must never answer for her nor listen partially to her answers by taking from the results of an experiment only those which support or confirm his hypothesis....

....if we are thoroughly steeped in the principles of the experimental method we have nothing to fear; for as long as the idea is correct, we go on developing it; when it is wrong experimentation is there to set it right.

If an hypothesis is not verified and disappears, the facts which it has enabled us to find are none the less acquired as indestructable materials for science.

Make experiments to seek the truth - not to destroy. The truely scientific spirit should make us modest and kindly.

Claude Bernard (1865).

Declaration.

This study was carried out under the guidance of Drs. J.H. Phillips and D.K. Apps at the Department of Biochemistry, University of Edinburgh Medical School between January 1983 and October 1985.

The experimental work presented in this thesis is my own. Where contributions from others is discussed this is acknowleged in the text.

The thesis presented herein has been composed by myself.

James G. Pryde

Department of Biochemistry,
University of Edinburgh
Medical School,
Hugh Robson Building,
George Square,
Edinburgh, EH8 9DS.

January, 1987.

Abstract.

An understanding of the biochemical processes underlying how secreted proteins and granule membrane proteins are delivered to, then post-translationally modified and assembled into secretory vesicles by the Golgi complex was the objective of this thesis. The bovine adrenal medulla whose secretory vesicles (called chromaffin granules) have been well characterised were the model chosen for this study.

1. **Subcellular fractionation** protocols were developed which yielded fractions enriched in enzyme markers for RER, Golgi and plasma membranes from a microsomal fraction. These fractions were characterised on two-dimensional electrophoretograms and nitrocellulose replicas of these were probed with antibodies to three major chromaffin granule proteins, and glycoproteins were identified by decoration with radioiodinated lectins.

2. **Primary cultures of chromaffin cells** were used to analyse the biosynthesis of the major secretory protein chromogranin A. Labelling studies with [$^{35}$S]-methionine identified secretory proteins synthesised and segregated into microsomal membranes as unglycosylated precursors from which a signal sequence had been removed. Depression of cytoplasmic ATP levels with the mitochondrial uncoupler FCCP prevented O-glycosylation suggesting that transport from RER to Golgi complex, where this modification takes place, was inhibited.

De novo synthesis of the integral membrane proteins DBH and cytochrome b561 was not detected in cultured cells, nor were their

precursors identified in subcellular fractions of tissue by immunoblotting analysis. Thus synthesis of secretory and membrane proteins was not concomitant. Lectin overlays did identify glycoproteins in RER which in contrast to those in Golgi and chromaffin granule membranes appeared to lack terminal sialic acid residues. These data support the idea that following exocytosis, chromaffin granule membranes are re-cycled to the trans region of the Golgi complex for re-use in the packaging of newly synthesised secretory proteins.

3. **Triton X-114** - this detergent was used to solubilise and phase separate membrane proteins into three groups: phospholipid-rich membranes produced a phase which separated spontaneously at $0^{o}C$; at $30^{o}C$ a detergent-rich phase was separated from the aqueous phase by centrifugation. Many integral membrane glycoproteins of the membrane were found in the latter phase, maintained in solution by residual detergent; when this was removed by dialysis these proteins precipitated and only truely hydrophilic proteins were left in solution. This protocol, developed using chromaffin granule membranes as a model, was used to simplify analysis of mitochondrial, plasma, Golgi and RER-enriched membrane fractions.

Acknowlegements.

I thank Dr. John H. Phillips and Dr. David K. Apps for the opportunity to study for this thesis and for their guidance and friendship over the years we worked together.

A special thanks to Lynn Kilpatrick, Alex Gray, and Judith Percy and many other members of the Department of Biochemistry, Edinburgh whose help, encouragement and friendship will not be forgotten. I thank Dr. Jeff Haywood for his help with fluorescence microscopy and photography and Mr. Sandy Purdie and everybody at the workshops for their patient and invaluable help.

I thank Dr. Susan Butler and members of the Department of Cardiology, University of Edinburgh Medical School, for carrying out phospholipid and cholesterol analyses.

I thank Dr. Monique Sensenbrenner and her colleagues at the Centre De Neurochimie Du CNRS, Strasbourg for their hospitality at the EPT regular course (November, 1984) "Cell Culture as a Tool for Research in Neurobiology", which was supported by the European Science Foundation.

I thank Dr. R. Kelly (University of California) and Dr. D. Meyer (EMBL, Heidelberg) for gifts of antibodies.

The work presented in this thesis was funded by a grant from the Medical Research Council.

I thank John Phillips for his criticism and suggestions regarding this manuscript.

Finally a special thankyou goes to Irene Pryde whose support, encouragement, understanding and belief in me over the years has made this thesis a reality.

Abbreviations.

ADP, adenosine 5'-diphosphate.
AMP, adenosine 5'-monophosphate.
AO, acridine orange.
Asn, asparagine.
ATP, adenosine 5'-triphosphate.
ATPase, adenosine 5'-triphosphatase.
Bq, Bequerels (1uCi=37Bq).
BSA, bovine serum albumin.
$C_{12}E_8$, octaethyleneglycol dodecylether.
CHO, Chinese hamster ovary.
CgA, chromogranin A.
Con A, concanavalin A.
Cytb561, cytochrome b561.
DBA, horse gram lectin.
DBH, dopamine $\beta$-hydroxylase.
mDBH, membrane bound form of DBH.
sDBH, soluble form of DBH.
DCCD, N,N'-dicyclohexylcarbodiimide.
DMEM, Dulbecos modified Eagles medium.
DTT, dithiothreitol.
EB, ethidium bromide.
EDTA, ethylenediaminetetra acetic acid.
EGTA, 1,2-di(2-aminoethoxy)ethane-N,N,N',N'-tetra acetic acid.
F12, Hams nutrient medium.
FCCP, carbonyl cyanide p-trifluoromethoxy phenyl hydrazone.
5FdU, 5-fluorodeoxyuridine.
Gal, galactose.
GalNAc, N-acetylgalactosamine.
GalNAcol, N-acetylgalactosaminitol.
$g_{av}$, relative centrifugal force.
GAV, Golgi associated vesicles.
GDP, guanosine diphosphate.
GERL, Golgi endoplasmic reticulum lysosome.
Glc, glucose.
GlcNAc N-Acetylglucosamine.
Gp, glycoprotein.
Hepes, N-2-hydroxyethylpiperizine-N'-2-ethane sulphonic acid.
HRP, horse-radish peroxidase.
IEF, isoelectric focusing.
LCL, lentil lectin.
Man, mannose.
MDCK, Madin-Darby canine kidney.
Mes, 2-(N-morpholino)ethane sulphonic acid.
Mops, 3-(N-morpholino)propane sulphonic acid.
$M_r$, relative molecular mass.
NADH, reduced nicotinamide adenine dinucleotide.
NADPase, nicotinamide adenine dinucleotide phosphatase.
NEM, N'-ethylmalaiemide.
NP40, Nonidet P40.
PAS, periodic acid Schiff reagent.
PCA, perchloric acid.

| | |
|---|---|
| pI, | isoelectric point. |
| PMSF, | phenylmethylsulphonyl fluoride. |
| PNA, | peanut agglutinin. |
| PSA, | *Pisum sativum* agglutinin. |
| RCA1&2, | ricin 1 and 2 agglutinins. |
| pnp, | p-nitrophenol |
| RNA, | ribonucleic acid. |
| SBA, | soybean agglutinin. |
| SDS, | sodium dodecyl sulphate. |
| SER, | smooth endoplasmic reticulum. |
| SFV, | Semiliki forest virus. |
| TBT, | tributyl tin chloride. |
| TCA, | trichloroacetic acid. |
| TEMED, | N,N,N',N'-tetramethyl-1,2-diaminoethane. |
| Tris, | tris-(hydroxymethyl)aminomethane. |
| UDP, | uridine diphosphate. |
| VSV, | vesicular stomatitis virus. |
| WGA, | wheat germ agglutinin. |

CONTENTS.

List of Tables.

List of Figures.

PUBLICATIONS.

The following publications contain work presented in this thesis:

1. Glycoproteins of the Chromaffin Granule Membrane: Separation by Two-Dimensional Electrophoresis and Identification by Lectin Binding.
   Gavine, F.S., Pryde, J.G., Deane, D.L., and Apps, D.K. (1984) J. Neurochemistry 43:1243-1252.

2. Isolation of ATPase I, the Proton Pump of Chromaffin-Granule Membranes.
   Percy, J.M., Pryde, J.G., and Apps, D.K. (1985) Biochem. J. 231:557-564.

3. Fractionation of Membrane Proteins by Temperature-Induced Phase Separation in Triton X-114.
   Pryde, J.G., and Phillips, J.H. (1986) Biochem. J. 233:525-533.

4. Triton X-114: a Detergent That Has Come in From the Cold.
   Pryde, J.G. (1986) Trends in Biochem. Sci. 11:160-163.

5. The Chromaffin Granule: A Model System for the Study of Hormones and Neurotransmitters.
   Phillips, J.H., and Pryde, J.G. (1986) Ann. N.Y. Acad. Sci. (in the press).

# CHAPTER ONE

# INTRODUCTION

## The Bovine Adrenal Gland

The bovine adrenal gland is composed of two types of tissue: the cells of the adrenal cortex, which surround the central medullary cells, specialise in the biosynthesis and secretion of steroid hormones, while the endocrine cells of the medulla secrete catecholamines, opioid peptides and a family of acidic glycoproteins in response to stress. The medullary (chromaffin) cells are highly modified post-ganglionic sympathetic neurons, that have differentiated from the cells of the ectoderm during their migration from the neural crest. Chromaffin cells are essentially an appendage of the sympathetic nervous system sharing many features in common with neurons. In primary culture they produce axon-like processes; this and their comparatively large secretory granule content has made them an attractive model for biochemical analysis. Unlike neurons, however, their secretory and biosynthetic machinery are not separated.

Intact chromaffin granules have a high buoyant density, while the membrane of the chromaffin granule is unusually rich in cholesterol and lipid; these properties have been exploited in differential and density gradient centrifugation, to provide preparations of both granules and membrane fractions of high purity. With the availability of large amounts of bovine material it is not surprising that biochemically the bovine chromaffin granule is probably the best characterised secretory granule. Such a characterisation makes the chromaffin cell an inviting model for studying the biogenesis of secretory and membrane proteins. In addition, the introduction in the late 1970's of primary cultures of chromaffin cells has made it possible to extend and improve earlier

studies, with perfused adrenal glands, on the biogenesis of their secretory proteins (Winkler et al., 1971).

The biogenesis of secretory granule components and their transport through the eukaryotic cell occurs within well-defined membrane compartments, newly synthesised secretory proteins undergoing post-translational covalent modifications within them. One such modification, the addition and subsequent processing of N-linked oligosaccharides on secretory glycoproteins, for example, is now understood in some detail and such studies, have helped elucidate the degree of compartmentation within the Golgi complex. This organelle is the subcellular crossroads for secretory and membrane protein traffic; it is here that routing and sorting decisions appear to be taken. Its study is therefore central to an understanding of intracellular protein processing.

Adrenal medullary cells synthesise secretory and membrane proteins at different rates, with apparently little turnover of membrane protein. This, in conjunction with similar evidence from other secretory systems, suggests that secretory granule membranes may be used for more than one round of secretion (Phillips et al., 1983). The discharge of secretory proteins to the extracellular space is an exocytotic process (Douglas, 1968). This requires that the secretory granule fuse with the plasma membrane. To maintain the integrity of the plasma membrane the cell must therefore retrieve secretory granule membrane. It does this by an endocytotic mechanism, with the retrieved membrane being returned to the trans region of the Golgi complex. These events in the life cycle of the secretory granule membrane are discussed in more detail below.

## The Project

The objective of the work described in this thesis was to extend our knowlege of the biochemical processes involved in the assembly of the chromaffin granule. Secretory granules arise from the transitional cisternae of the Golgi complex (Palade, 1975). Although this process has not been formally demonstrated in the case of the chromaffin granule, there is no reason to suppose that its biogenesis is any different from that of other secretory granules.

Dissection of the secretory pathway is possible because of the marked compartmentation of the post-translational modifications that occur to proteins during their biogenesis. Membrane-bounded compartments can be conveniently separated by centrifugation techniques. Fractions can be characterised by assaying for the enrichment in characteristic marker enzymes. The primary objective was therefore to isolate fractions enriched in rough endoplasmic reticulum and Golgi complex, then to identify within these fractions biogenic precursors to mature chromaffin granule proteins, probing for these with antibodies and with radioiodinated lectins, after their separation by two-dimensional electrophoresis. A concomitant approach was to use primary cultures of chromaffin cells to study the biosynthesis of the main secretory glycoprotein, chromogranin A. The effects of drugs known to disrupt glycosylation, were studied to try and localise the subcellular compartment in which glycosylation occurs.

A comprehensive review of the biogenesis of secretory proteins is beyond the scope of this thesis. However, in this introduction I will try to highlight the important concepts that have emerged over the last decade about the secretory pathway, with an emphasis on the Golgi complex and membrane recycling in particular. An in

depth review of the chromaffin literature has had to be omitted and only material relevant to granule assembly and secretion has been included. Where such omissions have been made the reader is referred to more comprehensive and erudite reviews and commentaries (Winkler, 1976,1977; Phillips and Apps, 1979; Winkler and Carmichael, 1980; Winkler et al., 1986).

**Protein Synthesis and Secretion.**

Animal cells specialising in the export of secretory proteins have an extensive reticular network of membranes associated with their nuclear envelope. Known as the rough endoplasmic reticulum (RER), these membranes are the site of synthesis of proteins destined for secretion. This reticular network however is not readily detected in electron micrographs of adrenal medullary cells. As will be discussed later this may not be surprising since these cells, unlike pancreatic cells for example, are not actively involved in the turnover of large amounts of secretory proteins (Coupland, 1965).

The RER is the first in a number of membrane-bounded compartments, through which secretory proteins pass during their post-translational life-time. The major stages along the secretory pathway in pancreatic cells were first delineated by Palade and his co-workers (Jamieson and Palade, 1968):

1. Synthesis
2. Segregation
3. Intracellular transport
4. Concentration
5. Intracellular Storage
6. Discharge

To this list we can now add two other stages, modifications

within the Golgi complex and membrane retrieval.

## Synthesis and Segregation

Secretory and membrane polypeptides are synthesised on ribosomes bound to the endoplasmic reticulum and are cotranslationally translocated across its membrane into the lumen. In many cases, ribosome binding and segregation of the nascent polypeptide depends upon the presence of an N-terminal extension of 15-30 amino acid residues (a 'signal'sequence). When this signal emerges from a free polysome it directs the interaction of the growing polypeptide (by now 60-70 amino acid residues long) with a 'signal recognition particle' (SRP) (Walter et al., 1981). The SRP causes a "site specific arrest of biosynthesis of the nascent protein" (Walter and Blobel, 1981,1983a). This precipitates a cycle of events, translation continuing once the arrested complex has interacted with a component on the endoplasmic reticulum, the SRP receptor (Gilmore et al., 1982a&b) or 'docking protein' (Meyer et al., 1982a&b). In vitro this 'arrest' does not appear to be a necessary prerequisite for translocation (Siegel and Walter, 1985).

During the elongation of integral membrane polypeptides, hydrophobic 'signal' domains prevent the complete transfer into the lumen of the RER experienced by secretory proteins (Blobel et al., 1979; Blobel, 1980; Sabatini et al., 1982). The role of these hydrophobic signals has been demonstrated by the use of recombinant DNA techniques to manufacture hybrids, in which addition of the transmembrane domain of an integral membrane protein converts a secretory protein into an integral membrane protein (Yost et al., 1983; Guan and Rose, 1984; Davis and Model, 1985). The segregation of secretory proteins within the lumen of the RER is an irreversible

step, presumably brought about by folding of the polypeptide and co-translational modifications such as disulphide bond formation, limited proteolysis and the addition of N-linked oligosaccharide cores.

Following their synthesis and modification in the RER proteins must be transported to the Golgi complex before further post-translational modifications can occur.

## Intracellular Transport and Sorting of Secretory and Membrane Proteins.

### 1. Transport from the RER to the Golgi Complex.

Morphological studies suggest that the intracellular movement of secretory proteins is mediated by vesicular translocations (Jamieson and Palade, 1968). The molecular mechanisms of both this process and the sorting of proteins are unknown. It is clear however that there are a number of important steps controlling transport. One of these is the prevention of further translation of polypeptides destined for the RER, by the signal recognition particle in the cytoplasm. Another is the transport of material from the RER to the cis-face of the Golgi complex, an energy dependent process requiring ATP (Harwood et al., 1976). After traversing the Golgi complex secretory proteins finally exit from its trans-face, and it is at this point that decisions about the final destinations of proteins appear to take place. The 'sorting' of secretory and integral membrane proteins appears to be tightly coupled to these translocation events.

An observation that provided important clues as to how cells sort and transport proteins was the recognition that liver and hepatoma cells, among others, synthesising two (Morgan and Peters,

1971; Strous and Lodish, 1980) three (Ledford and Davis, 1983) four (Fries et al., 1984) or five (Lodish et al., 1983) proteins for secretion, discharge them at different rates. This was indicative of a selective process rather than the bulk transport of protein pools. These differential rates of transfer were linked to control of the transfer of secretory proteins from the RER to the cis-face of the Golgi complex. The intracellular transport of membrane glycoproteins also appeared to be controlled in a similar way (Strous and Lodish, 1980; Fitting and Kabat, 1982), rather than there being different pathways to the Golgi complex for integral membrane proteins and secretory proteins (Strous et al., 1983a; Strous and Lodish, 1980).

The differential transport of the membrane glycoproteins of murine leukemia virus (Fitting and Kabat, 1982) suggested that the process was selective, and not just a passive transfer of material. There was also a concentration or selection of albumin, at regions of the RER from which transport occurred (Yokota and Fahimi, 1981). Thus a signal structure on proteins may influence the rate at which proteins leave the RER. Covalent post-translational modifications may provide such a signal for recognition by receptor molecules on the membrane of the RER, directing secretory proteins for packaging into transport vesicles whose cargo is bound for the Golgi complex. Such signals may include part of the primary or even the tertiary structure of the protein. Even small changes in the primary structure of a protein, such as the substitution of a single amino acid residue at a critical position in a sequence (Wu et al., 1983) can change the rate of secretion. Many, but not all, glycoproteins fail to be secreted if glycosylation is prevented by treatment with tunicamycin; they may become more sensitive to proteolysis (Olden et

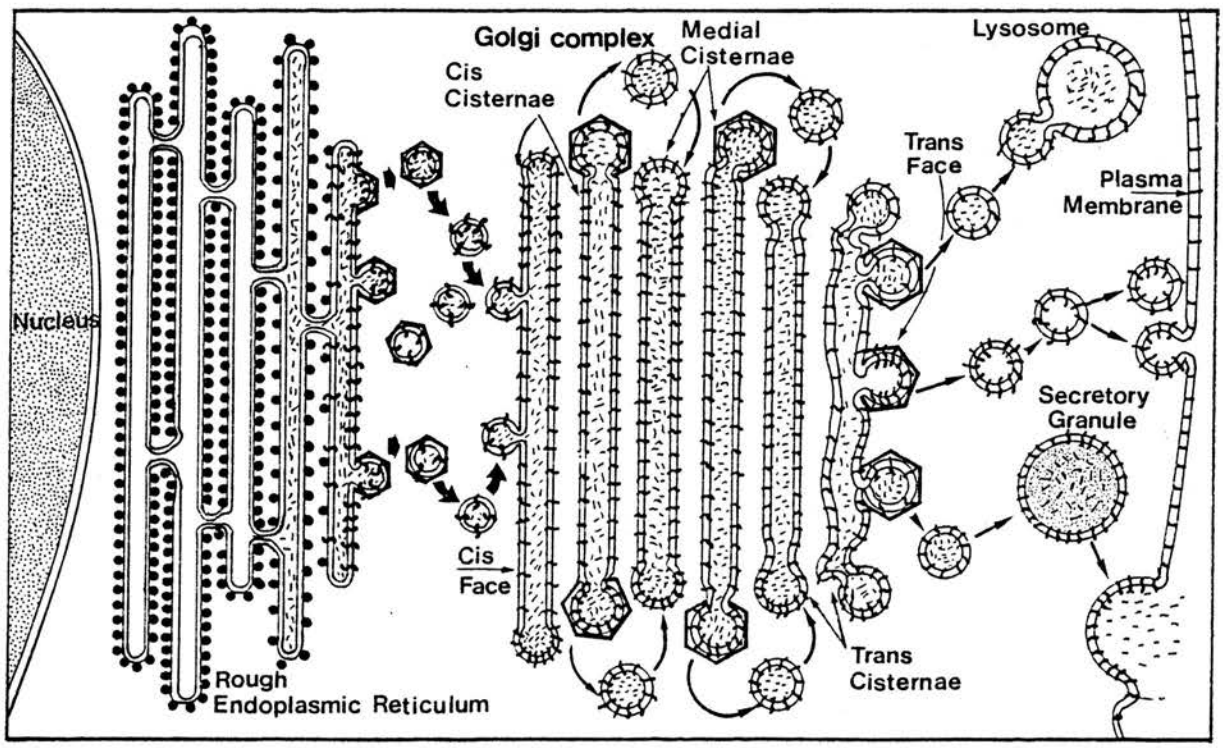
al., 1978; Schwarz et al., 1976), or become less soluble (Gibson et al., 1980; Tarentino et al., 1974) or may even be abnormally folded (Wang and Hirs, 1977). Efficient maturation of the 'signal' may even require more subtle modifications, such as the removal of the 'capping' triglucosyl residue from the cores of N-linked glycoproteins to reveal the $Man_9GlcNAc_2Asn$ core (see below) a putative signal for such a receptor (Lodish and Kong, 1984; Reitman et al., 1982; Gabel and Kornfeld, 1982). The only piece of hard evidence for signal receptor theories of this sort is that the transfer of some acid hydrolases from the Golgi complex to lysosomes does appear to require that a 6-phosphomannosyl signal be attached to the asparagine-linked oligosaccharides (Distler et al., 1979; Natowicz et al., 1979; Rome et al., 1979).

## 2. Morphology of the Golgi Complex

Electron micrographs of both exocrine and endocrine cells show the Golgi complex as a collection of four or five semicircular membrane bounded compartments. Called cisternae, they are stacked together within a 'zone of exclusion' devoid of other organelles (see Figure 1.1). The compartment at the cis-face of the plate-like cisternal stack is presented to the nucleus and the rough endoplasmic reticulum and accepts the cargoes of newly synthesised proteins which have been packaged and transported in vesicles whose membranes have been derived from the transitional membrane elements of the RER (Farquhar and Palade, 1981).

The membranes of the Golgi stack are more enriched in phospholipids and cholesterol than those of the RER. Early cytological studies showed differential patterns of staining with dyes and precipitates which hinted at possible biochemical

FIGURE 1.1 The Secretory Pathway.




This representation of the secretory pathway shows the endomembrane compartments encountered by both secretory proteins and integral membrane proteins following their synthesis on ribosomes bound to the endoplasmic reticulum. During their translation into the lumen of the endoplasmic reticulum many secretory proteins undergo some co-translational modifications to their primary structure. Before any major post-translational modifications occur the proteins must be transported to the cis-face of the Golgi complex within protein coated vesicles, a process which can be shown to be energy dependent. These transport vesicles, once they have given up their protein coats, fuse with the cis-cisternal compartment and donate their protein cargoes. Both secretory proteins and integral membrane proteins then appear to undergo coated-vesicle mediated transport between the Golgi compartments, being modified and sorted before leaving from the trans face of the Golgi complex en route for either the plasma membrane, lysosomes or, in cells with a regulated secretory pathway, for storage in secretory granules prior to secretion.

Rough Endoplasmic Reticulum; Integral Membrane Proteins; Secretory Proteins; Transport Vesicles.

differences between the cisternae. For example, the prolonged treatment of cells with osmium tetroxide led to the deposition of a precipitate of osmium in one or two cisternae at the cis-face of the Golgi complex (Friend and Murray, 1965; Fleischer et al., 1969), while a UDPase activity (thiamine pyrophosphatase) appeared restricted to the trans Golgi cisternae (Cheetham et al., 1971). An NADPase activity is also restricted to one or two cisternae in the centre of the Golgi complex (Smith, 1980).

It was differential staining of Golgi cisternae by periodic acid-Schiff reagent (Rambourg and Leblond, 1967) that suggested that the morphological and cytochemical heterogeneity shown by the Golgi complex may be related to the synthesis of the complex carbohydrate moieties on glycoproteins (Neutra and Leblond, 1966). Binding studies with lectins (Griffiths et al., 1982; Tartakoff and Vassalli, 1983) now suggest that there are at least three compartments within the Golgi complex: cis, medial and trans (see Figure 1.1), each involved in the post-translational modification of proteins (see Table 1.1). Secretory proteins having arrived at the cis-face of the Golgi complex now present it with the formidable task of modifying and sorting them for delivery to specific compartments within the cell.

### 3. Cis-face of the Golgi.

Studies with cells infected with enveloped viruses have shown that after leaving the endoplasmic reticulum, membrane glycoproteins pass through the cis-face of the Golgi complex (Bergmann et al., 1981; Bergeron et al., 1982; Green et al., 1981; Griffiths et al., 1982; Rindler et al., 1982). However, it now appears that before crossing this boundary the glycoproteins of viral envelopes and the

Table 1.1. The Golgi Complex: Distribution of Functions, and the Enzymes Involved in Post-Translational Modifications.

Cis-Cisternae.

Acetylation: the covalent attachment of fatty acids.

GlcNAc phosphotransferase.

Phosphoglucosidase.

Medial-Cisternae.

Mannosidases Ia and Ib.

GlcNAc Transferase I.

Mannosidase II.

GlcNAc Transferases II (III&IV).

Fucosyltransferase.

Trans-Cisternae.

Galactosyltransferase.

Sialyltransferase *.

Sulphation.

Phosphorylation *.

Proteolytic processing of proproteins *.

Functions not Localised.

Amidation.

Formation of Lysosomes.

Sorting of Proteins *.

Assembly of Secretory Vesicles *.

*-these functions may also occur in the trans reticular network.

glycoproteins of secretory cells encounter an intermediate 'organelle' in this proximal region of the Golgi complex; a Golgi-associated-vacuole (GAV) (Saraste and Hedman, 1983; Saraste and Kuismanen, 1984). These vacuoles were observed during experiments with temperature-sensitive mutants of influenzae virus (in MDCK cells) and Semliki forest virus (SFV) (in fibroblasts), in which reversible temperature-induced blocks in protein transport could be created. At 15°C a block at the cis-face of the Golgi was formed, with radiolabelled viral envelope glycoprotein accumulating in 300-400nm vacuoles, with very little of the label in the Golgi stacks. This suggested that entry into the cis-face had been inhibited (Saraste and Kuismanen, 1984). A population of 50nm protein-coated vesicles was apparently responsible for shuttling labelled protein from the RER to these GAV's. These donor vesicles gave rise to smooth vesicles, their protein coats dissociating, prior to fusion with the target GAV's (Saraste and Hedman, 1983; Altstiel and Branton, 1983; Rothman et al., 1982). Incubation at 20°C arrested intracellular transport of the viral glycoproteins at a terminal Golgi location (Matlin and Simons, 1983) and led to an accumulation of SFV glycoprotein in 80nm vacuoles at the trans region of the MDCK cell or fibroblast Golgi complexes (Saraste and Hedman, 1983; Saraste and Kuismanen, 1984).

### 4. Intracellular Transport.

The membrane interactions involved in the two way transport of proteins between the Golgi complex and plasma membrane are mediated by clathrin coated vesicles (Palade, 1975; Griffiths et al., 1982). We have also seen that vesicles with cytoplasmic protein coats appear to be involved in the sorting and transport processes that

take place between the transitional elements of the endoplasmic reticulum and the cis-face of the Golgi complex (Figure 1.1). The nature of this coating protein remains to be demonstrated. What do we know about the events of the transport process itself?

Transport between Golgi stacks mediated by protein-coated vesicles relies on two events; first, the budding of membranes from the dilated rims of the cisternal donor compartment, followed by a dissociation into the surrounding 'cytoplasm' (fission), and second, the subsequent interaction with putative receptors on an acceptor compartment (fusion) (Rothman et al., 1984a&b). This mechanism of transfer between Golgi stacks has been studied in Chinese hamster ovary (CHO) cells in which a clone (15B) of cells deficient in N-acetylglucosaminyltransferase I was fused with wild type cells capable of adding N-acetylglucosamine to vesicular stomatitis virus (VSV) G-protein. Transfer of this viral envelope protein was found to be a "dissociative and vectorial process" (Rothman et al., 1984a&b). This transfer has since been studied in a cell-free assay and by electron microscopy (Balch et al., 1984a&b; Braell et al., 1984; Orci et al., 1986).

Intracellular transport of proteins to the plasma membrane, endocytosis and phagocytosis cease when a cell begins to divide (Warren et al., 1983; Berlin and Oliver, 1980; Berlin et al., 1978). This suggests that membrane fission and fusion processes are not compatible with the events involved in the reorganisation of membranes at this time. The cessation of vesicle-mediated transport during mitosis is accompanied by the apparent fragmentation of the Golgi complex into tens of thousands of small vesicles (Burke et al., 1982; Zeligs and Wollman, 1979). An attractive hypothesis is that dispersion of these vesicles would ensure that at telophase

each daughter cell would receive an equal complement of Golgi membranes. There is also a concomitant cessation of the fusion of secretory granules with the plasma membrane (Hesketh et al., 1984) and of the internalisation of membrane vesicles from the cell surface (Burke et al., 1982).

## Post Translational Modification of Secretory and Membrane Proteins.

### 1. Glycosylation

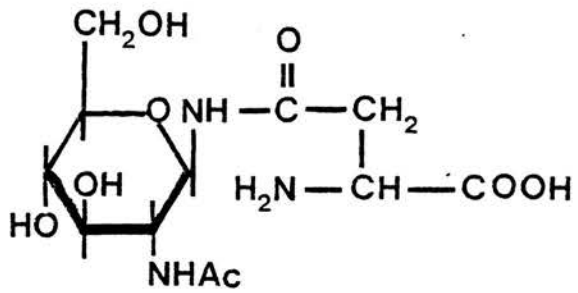
There are two major types of carbohydrate-peptide linkage to proteins (see Figure 1.2). One is the co-translational addition of an oligosaccharide moiety by an N-linkage to asparagine, which can undergo extensive post-translational modification. The other, which is discussed briefly later, is the O-glycosidic linkage of sugars to serine or threonine, a post-translational modification which also appears to occur in the Golgi complex.

The biogenesis of the N-linked glycoproteins has been studied in Chinese hamster ovary (CHO) cells, infected with vesicular stomatitis virus (VSV). Viruses "hi-jack" a cell's biosynthetic pathway normally used for the modification, transport and packaging of secretory proteins, and use it to manufacture their own viral envelope protein. The VSV envelope protein, the G-protein, is an integral membrane glycoprotein. Its oligosaccharides are N-linked to asparagine and are of the high-mannose type (see Figure 1.3). Studies with viral glycoproteins and secretory proteins containing N-linked oligosaccharide residues have shown that the pathway and the enzymes involved in the biogenesis of these glycoproteins are common to all eukaryotic cells studied so far. The fact that viruses can subvert the glycosylation machinery of many cell types

FIGURE 1.2 CARBOHYDRATE-PEPTIDE LINKAGES TO GLYCOPROTEINS

N-GLYCOSIDIC.

β-N-Acetylglucosaminyl-asparagine




O-GLYCOSIDIC

α-N-Acetylgalactosaminyl-serine/threonine

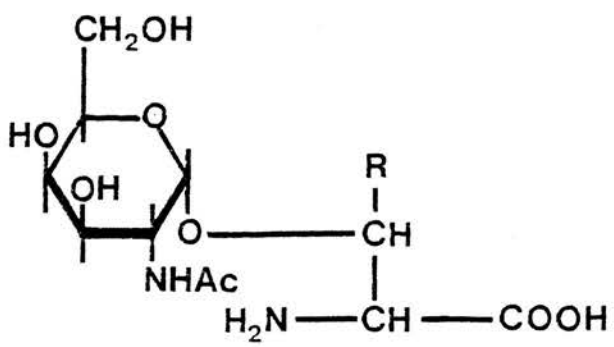
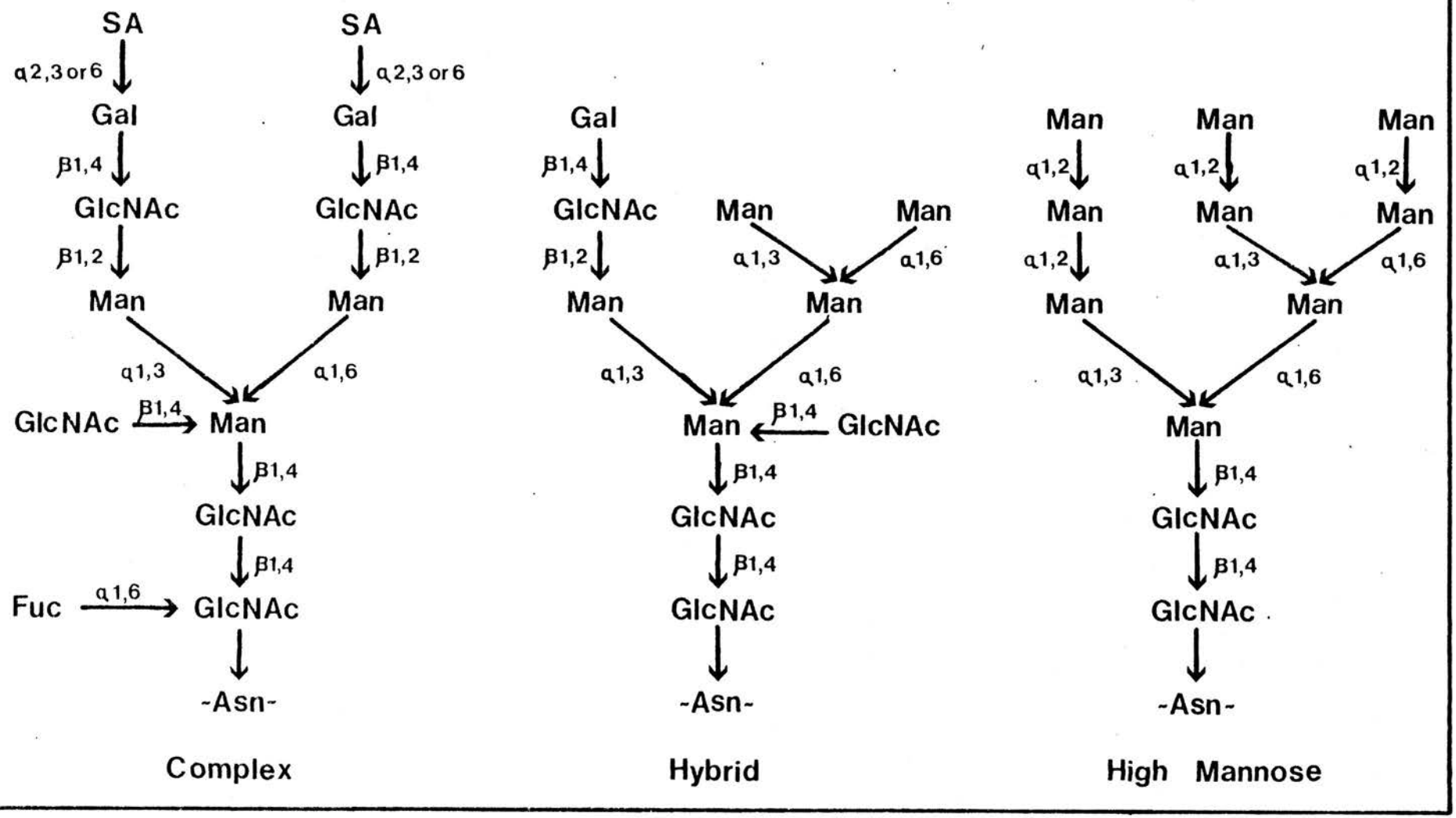


FIGURE 1.3
Structures of the Main Types of Asparagine-Linked Oligosaccharides



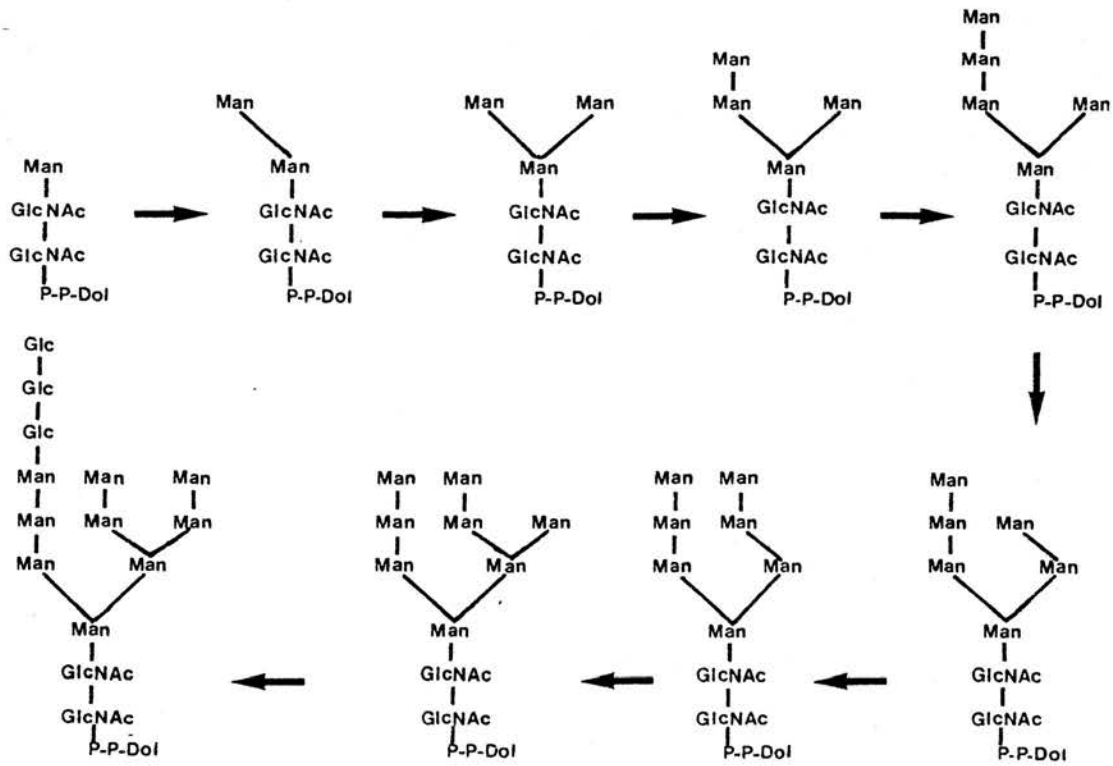
tends to confirm the ubiquitous nature of this pathway. Therefore the pathway for the N-glycosylation of the adrenal medullary enzyme dopamine $\beta$-hydroxylase should not be significantly different from that outlined below for other N-linked glycoproteins.

2. RER: The High Mannose Core.

The addition of a "high-mannose core" to proteins is a co-translational event. This oligosaccharide core is constructed by the sequential addition of sugars to a carrier lipid moiety, dolichol pyrophosphate (see Figure 1.4). Within the lumen of the RER an oligosaccharyl transferase then catalyses an en bloc transfer of the oligosaccharide from its carrier and attaches it through an N-linkage to an asparagine residue on the growing polypeptide chain (see Figure 1.5) (Hanover et al., 1980; Lingappa et al., 1978; Katz et al., 1977; Snider and Robbins, 1982). Pools of the mature oligosaccharide-lipid intermediate have been identified on the luminal side of the RER (Snider and Robbins, 1982), but how the nucleotide-sugar precursors (Coates et al., 1980) cross the RER membrane for its assembly is not clear. All the enzymes involved in the synthesis of the lipid-linked oligosaccharide appear to be associated with the RER membrane (Snider and Rogers, 1984). Studies, in which the trans-membrane orientation of oligosaccharide-lipid intermediates have been probed with concanavalin A (which binds primarily to mannose residues), suggest an assembly process in which the intermediate $Man_5GlcNAc_2$-lipid (see Figure 1.4) is assembled on the cytoplasmic face of the RER membrane, before being transported to the luminal face where the $Glc_3Man_9GlcNAc_2$-lipid unit is completed (Snider and Rogers, 1984).

However the demonstration of UDP-GlcNAc transport across the

FIGURE 1.4. The Assembly of the High-Mannose Core.




This figure shows the proposed sequential addition of mannose residues to dolichol pyrophosphate-GlcNAc$_2$Man in the assembly of the high-mannose core. The final seven sugars are added from the nucleotide precursors UDP-GlcNAc and GDP-Man. The final four mannose residues and the three 'capping' glucose residues are added from dolichol phosphate-mannose and dolichol phosphate-glucose moieties. This figure has been adapted from Hubbard and Ivatt (1981).

membrane of the RER (Perez and Hirschberg, 1985) suggests that the construction of $GlcNAc_2$-P-P-dolichol may occur on the luminal side of the RER membrane. Translocation of the companion nucleotide-sugars UDP-glucose and GDP-mannose has yet to be demonstrated.

The 'triglucosyl cap' is co-translationally removed from the nascent glycopeptide by two membrane bound glycosidases to generate the $Man_9GlcNAc_2$-Asn high mannose core (see Figure 1.5) (Atkinson and Lee, 1984). Glucosidase I removes the outer $\alpha$-1,2-linked glucose residue (see Figure 1.5), while the inner two $\alpha$-1,3-liked glucose residues are removed by glucosidase II (Hubbard and Ivatt, 1981). The function of the "triglucosyl cap" is unknown, but it may protect the dolichol-phosphate-oligosaccharide moiety from degradation (Hoflack et al., 1981). Its removal is obligatory however, if processing of the mannose residues is to proceed and we have already seen that its removal may function as a signal for targeting the glycoprotein during transport out of the RER to the Golgi. Before arriving at the Golgi-complex, however, some preliminary processing of mannose residues on the high mannose core may have taken place (see Figure 1.5). A $Man_8GlcNAc_2$Asn species has been detected in RER membrane fractions of thyroid slices (Godelaine et al., 1981) and $Man_8$-, $Man_6$- and $Man_5$-$GlcNAc_2$Asn species have also been detected in liver RER fractions (Hercz and Harpaz, 1980). These findings suggest that mannose trimming of secretory glycoproteins may well start before the Golgi complex and an $\alpha$-1,2-mannosidase activity has been identified in rat liver RER (Bischoff and Kornfeld, 1983). These trimmed high mannose core structures may be re-glucosylated by an RER glucosyltransferase (Parodi et al., 1984).

FIGURE 1.5. Asparagine-Linked Oligosaccharide Processing Pathway.

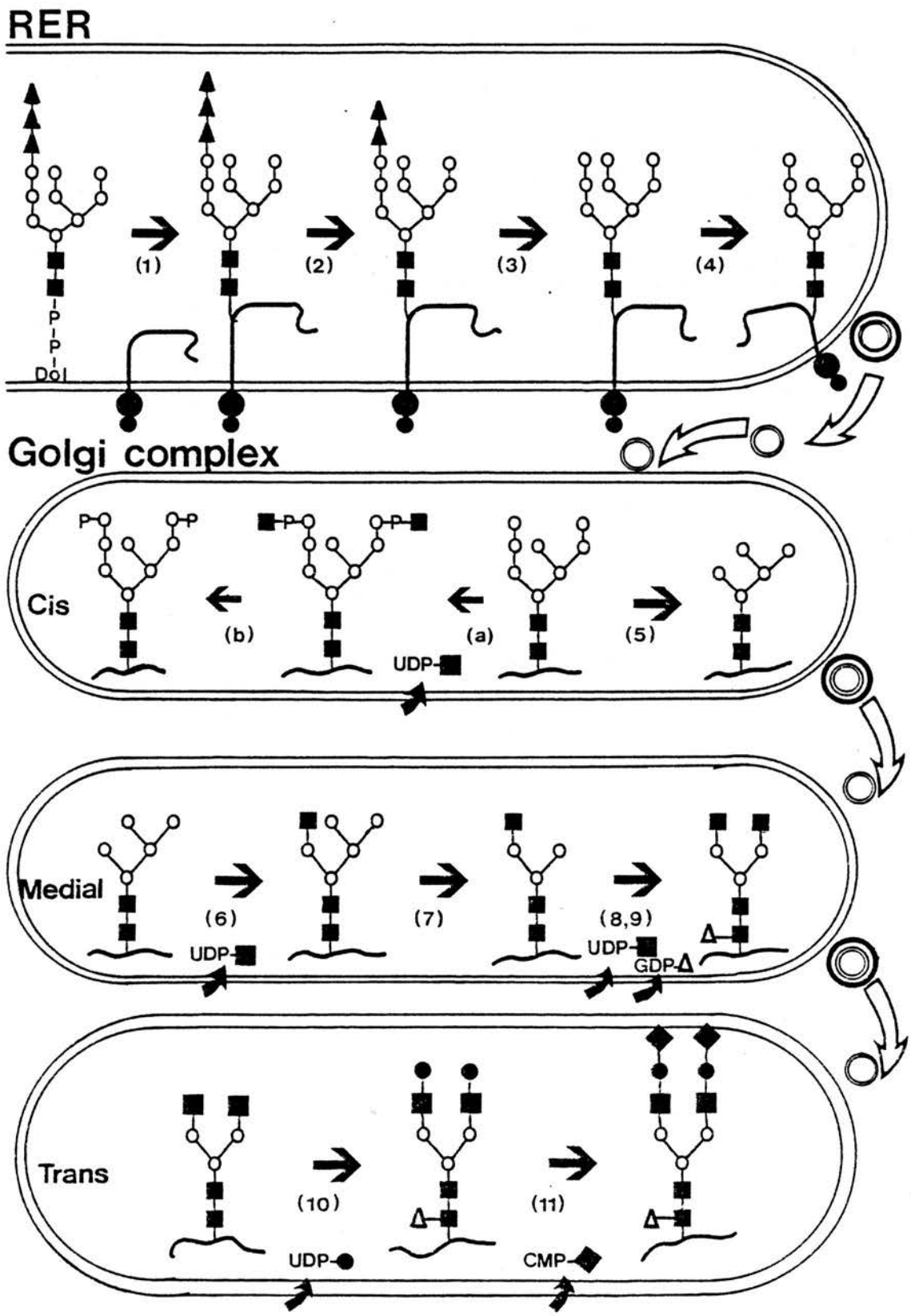
This figure is a representation of the modifications to Asn-linked oligosaccharides on newly synthesised secretory and membrane glycoproteins, which take place initially in the RER and later in the stacked cisternal elements of the Golgi complex to which they are transported in coated-vesicles (◎-coated; o-uncoated).

After the cotranslational addition of the oligosaccharide moiety from dolicholpyrophosphate (Dol-P-P) by an oligosaccharyltransferase (1) the modifications are catalysed by the following sequence of enzymes: ∝-glucosidase I (2), ∝-glucosidase II (3), ER 1,2-mannosidase (4), N-acetylglucosaminyl phosphotransferase (a), N-acetylglucosamine-1-phosphodiester -N-acetylglucosaminidase (b), [ (a) and (b) represent modifications to lysosomal proteins], Golgi ∝-mannosidase I (5), N-acetylglucosaminyltransferase I (6), Golgi ∝-mannosidase II (7), N-acetylglucosaminyltransferase II (8), fucosyltransferase (9), galactosyltransferase (10), sialyltransferase (11).

The symbols represent: ▮ ,membrane-bound ribosome; ■ , N-acetylglucosamine; o , mannose; ▲, glucose; P, phosphate; ▲, fucose; ●, galactose; ◆, sialic acid.

This figure has been adapted from Kornfeld and Kornfeld (1985).

FIGURE 1.5 ASPARAGINE-LINKED OLIGOSACCHARIDE PROCESSING PATHWAY.



3. Cis and Medial Golgi Cisternae.

The processing of $\alpha$-1,2-linked mannose residues, occurs in the medial cisternae of the Golgi complex (Table 1.1). Golgi $\alpha$-mannosidase Ia (Tartakoff, 1980) and Golgi $\alpha$-mannosidase Ib (Tulsiani et al., 1982) between them produce a $Man_5GlcNAc_2Asn$ core (details in Figure 1.5). Mannose trimming may occur in this compartment to prevent competition between the Golgi-mannosidases and GlcNAc-phosphotransferase for $\alpha$-1,2-linked mannose residues. GlcNAc-phosphotransferase (Reitman et al., 1982; Waheed et al., 1981) and phosphoglycosidase are located in the cis-cisternae of the Golgi (Varki and Kornfeld, 1981; Waheed et al., 1981). Between them these enzymes generate the Man-6-P recognition marker for targeting some lysosomal proteins (see below).

Compartmentation within the Golgi complex appears to be necessary for control of the assembly of complex oligosaccharides. Furthermore, the complex variety of modifications to N-linked glycoproteins which takes place in the several medial Golgi stacks suggests that an even more complex compartmentation may be involved in the control of processing. However, at present the biochemical tools for probing such functions lack the resolution necessary for further dissection.

Following the removal of the outer $\alpha$-1,2-linked mannose residues the $Man_5GlcNAc_2$-Asn core becomes the substrate for a number of glycosyltransferases within the medial Golgi cisternae. N-acetylglucosaminyl transferases I&II initiate the construction of branching which is typical of the complex glycoproteins, by the addition of GlcNAc residues (see Figure 1.5). For example the biantennary structures found on VSV G-protein and on dopamine $\beta$-hydroxylase (Figure 1.3) and more complex "bisected" structures

are produced by GlcNAc-transferase III which adds an $\beta$,1,4-linked GlcNAc to the innermost $\beta$-linked mannose residue (Hubbard and Ivatt, 1981). When this enzyme acts before GlcNAc-transferase II a "bisected hybrid" results due to the inhibition of mannosidase II processing (Harpaz and Schachter, 1980).

Chinese hamster ovary cell mutants deficient in N-acetylglucosaminyltransferase I accumulate $Man_5GlcNAc_2Asn$. This suggests that before the two inner mannose residues can be removed by Golgi $\alpha$-mannosidase II (see Figure 1.5), the addition of N-acetylglucosamine by GlcNAc-transferase I is obligatory. One result of this processing is to make the glycoprotein resistant to endoglycosidase H, which previously could cleave the oligosaccharide between the inner two GlcNAc residues. This differential response to endoglycosidase H has been exploited to study the temporal nature of transport from the RER to the Golgi (Tarentino and Maley, 1974)

Branching on the oligosaccharide is further controlled by the addition of a galactose residue to the GlcNAc residue on the $\alpha$-1,3-mannose branch, and this can be terminated by the addition of sialic acid residues to the terminal galactose residues. The addition of galactose by galactosyltransferase, in the trans Golgi cisternae, prevents further processing by at least five enzymes: Golgi-mannosidase II, GlcNAc-transferases II, III and IV, and also by an $\alpha$-1,6-fucosyltransferase. Thus the segregation of the galactosyl transferase to the trans-cisternae may prevent premature interruption of GlcNAc-transferase processing.

Post-translational modification of glycoproteins is only one of the major functions of the Golgi complex. But, by studying the enzymes involved in glycosylation some understanding of the

compartmentation within the stacks has been achieved. However, it is hard to envisage that compartmentation is only a reflection of the distribution of the glycosidases and glycosyltransferases. Cytoplasmic enzymes do not appear to require such compartmentation, relying instead on their substrate specificities to enable a sequence of reactions to remain ordered. However, the complexity and diversity of structures on glycoproteins may demand the spatial and temporal segregation provided by compartmentation.

### 4. O-glycosylation.

The Golgi complex is the location for many other post-translational modifications (Table 1.1). While a great amount of effort has been put into elucidating the process of N-linked glycosylation relatively little work has been done to characterise the pathway of O-glycosylation. However, this modification is especially relevant to studies with adrenal medullary cells as the major secretory glycoprotein, chromogranin A, has all its carbohydrate O-glycosidically linked (Kiang et al., 1982; Apps et al., 1985; Benedum et al., 1986). The biogenesis of this protein will be discussed in Chapter 6.

Within the Golgi complex N-acetylgalactosamine is transferred directly from UDP-GalNAc to the hydroxyl group of serine and/or a threonine residue (Hanover and Lennarz, 1981) without the intervention of any preassembled oligosaccharide or lipid-linked intermediate (Roth, 1984). Sugars are then added sequentially: galactose, sialic acid, fucose and in some cases GalNAc (Schachter et al., 1971; Beyer et al., 1979). It is likely that the elongation of O-linked oligosaccharide chains occurs sequentially within the medial and trans cisternae of the Golgi in a manner similar to their

N-glycosidically linked cousins (Schachter and Roseman, 1980). The envelope glycoproteins of animal viruses were instrumental in the deciphering of the pathway of N-glycosylation. While there are relatively few O-glycosidically linked viral glycoproteins the biosythesis of one, coronavirus glycoprotein E1, was shown to be uninhibited by tunicamycin (Niemann et al., 1982), while monensin blocked the addition of its O-linked sugars without affecting N-glycosylation (Niemann et al., 1982; Johnson and Spear, 1983). This was indicative of the Golgi being involved in this modification; N-acetylgalactosaminyltransferase appears to be present only in smooth membrane fractions and not in rough microsomes.

## 5. Modifications Within Cis- Golgi Cisternae.

### a. Sorting of Lysosomal enzymes.

The enzymes for generating the mannose-6-phosphate recognition marker on lysosomal acid hydrolases, GlcNAc-phosphotransferase (Reitman et al., 1982; Waheed et al., 1981) and phosphoglycosidase, are located within the cis cisternae of the Golgi complex. It has been postulated that those proteins destined for lysosomes, are sorted from the bulk of newly synthesized protein by receptor molecules. These receptors have been localised by immunocytochemical techniques to one or two cisternae at the cis-face of the Golgi stack, and also to coated vesicles, endosomes and lysosomes. From this localisation it has been postulated that lysosomal enzymes are ferried from the cis-face in protein-coated vesicles to the lysosomes (Brown and Farquhar, 1984). This idea however does not agree with the more established view that lysosomal enzymes traverse the Golgi stack and exit from the trans-face

(Rothman, 1981; Goldberg and Kornfeld, 1983). Nor is it consistent with the suggestion that lysosomal enzymes are transported directly to lysosomes from a specialised compartment of the endoplasmic reticulum "GERL", which is closely apposed to the trans cisternae of the Golgi thus by-passing the majority of the Golgi stack, with new lysosomes budding from the GERL (Novikoff et al., 1971; Novikoff, 1976).

b. Acylation.

The addition of fatty acid residues to proteins occurs in the Golgi complex (Schmidt and Schlesinger, 1980; Schmidt et al., 1979). The covalent attachment of [$^3$H]-palmitate to VSV G-protein has been demonstrated in pulse and chase studies with cells and the modification was localised to the cis-face of the Golgi complex (Dunphy et al., 1981; Quinn et al., 1983); the lipid donor was palmitoyl-CoA (Berger and Schmidt, 1984).

Acylation of proteins is a widespread modification and two types of fatty acid-protein linkage have been described. The addition of palmitate ($C_{16:0}$) is through a hydroxylamine-sensitive thioester linkage to the side chain of cysteine residues. The other is an amide linkage to the amino terminal of proteins, which is insensitive to hydroxylamine cleavage and is exclusive to myristic acid ($C_{14:0}$) (Magee and Courtneidge, 1985). The function of these modifications is not known, but the presence of covalently attached fatty acid may augment the hydrophobic nature of integral membrane proteins, helping them to remain anchored to their membrane as they are sorted and transported through-out the cell (Schmidt and Schlesinger, 1980).

6. Modification and Exit from the Trans-Golgi Cisternae.

The best understood modification to proteins within this Golgi compartment is the addition of terminal galactose residues to GlcNAc and capping with sialic acid residues. The transferases involved have been localised to the trans-Golgi cisternae by immuno-labelling and decoration with protein A-gold (Roth and Berger, 1982; Roth et al., 1985); galactosyltransferase is widely used as a Golgi marker. This compartment is also responsible for at least two other important post-translational modifications, sulphation and the proteolytic processing of proproteins. This latter modification is tightly coupled to the exit of proteins from the trans-cisternae and appears to continue within clathrin-coated secretory granules (Orci et al., 1985; Tooze and Tooze, 1986).

a. Sulphation

Covalently bound sulphate groups have been detected on both carbohydrate structures and O-linked to the tyrosine residues of many proteins (Huttner, 1982). Electron microscopic and pulse chase studies with the radiolabelled sulphate precursor adenosine 3'-phosphate 5'-phosphosulphate (PAPS), which is translocated into the Golgi complex (Schwarz et al., 1984), have shown sulphate transfer by a specific sulphotransferase (Young, 1973) and covalent binding to carbohydrate residues on for example glucosaminoglycans and chondroitin (sulphate). Although the exact location of the sulphotransferases within the Golgi is unknown, the addition is probably distal to or in the same compartment as sialic acid addition. The sulphotransferase responsible for O-sulphation of tyrosine residues has not been identified. Several proteins with known biological activities, such as fibrinogen and complement protein C4, for example, contain this linkage (Huttner, 1984). It

has been suggested that the role of tyrosine sulphation may not lie in its effects on the functional properties of proteins after secretion, but rather in affecting the way proteins are secreted i.e. acting as a signal (Baeuerle and Huttner, 1984). Sulphation has been generally implicated in the control of cell growth and differentiation.

b. Processing of Prosecretory Proteins.

Secretory proteins are packaged within secretory vesicles in the trans region of the Golgi complex. Many secretory proteins are synthesised as inactive proproteins which require proteolytic processing before becoming biologically active. Locating the precise site of this modification has been difficult. However, current evidence suggests that a Golgi compartment distal to the TPPase positive trans cisterna (the trans Golgi network) may be involved (see Tooze and Tooze, 1986).

Proteolytic processing has been associated with the Golgi complex ever since the demonstration that inhibitors, which blocked the energy dependent transport of proinsulin from the RER to the Golgi complex, also prevented its conversion to insulin (Orci et al., 1984). Monensin, which disrupts the trans region of the Golgi complex (Tartakoff, 1983), also prevents this proteolytic conversion. However, in addition to the dilation of the trans cisternae and the concomitant inhibition of protein transport from the Golgi stack (Tartakoff, 1983) a population of clathrin coated vesicles undergo alkalinisation when $\beta$-pancreatic cells are treated with monensin (Orci et al., 1984). While bearing in mind that monensin may cause many other effects within the cell, it is possible that proteolysis, probably the action of a thiol-dependent protease with an acidic pH optimum (Docherty et al., 1982), is

inhibited by the monensin-induced rise in pH. Similar inhibitions of proteolytic processing of proproteins have been described for many cell types in the presence of ionophores (Orci et al., 1984).

Recent immunocytochemical evidence suggests that protein-coated immature secretory vesicles may be the major site of the conversion of proinsulin to insulin (Orci et al., 1985) and that the Golgi cisternae may not be involved in this processing. Ultrastructural studies do suggest however that de novo assembly of secretory granules is mediated by a population of coated vesicles which bud from the trans cisternae of the Golgi complex (and presumably includes secretory granule membrane returning from the plasma membrane; discussed by Kelly, 1985; Tooze and Tooze, 1986).

The Golgi-associated proteases have not been purified and their precise location is unknown. The endogenous processing enzymes for all proproteins appear to be similar as their substrates all contain paired basic residues (Arg-Arg or Lys-Arg) at the cleavage site. In vitro, proinsulin can be converted to insulin and proparathyroid hormone to parathyroid hormone by the combined action of an endopeptidase and an exopeptidase (Steiner et al., 1984).

Origin and Turnover of Secretory and Membrane Proteins.

The Golgi complex is involved in the post-translational modification of secretory granule membrane proteins. Studying the biogenesis of endogenous membrane proteins and the assembly of granule membrane has been problematical. Only indirectly has the Golgi complex been shown to be involved in granule assembly. Many secretory granule membrane proteins are however glycoproteins, which implies that they pass through the Golgi complex for addition of terminal sugars (Farquhar and Palade, 1981). The biogenesis of

viral envelope glycoproteins, in particular the use of temperature-sensitive mutants, has enabled the role of the Golgi complex in glycosylation to be outlined (Bergmann et al., 1981).

Secretory granule biogenesis poses two questions: 1. Is new granule membrane synthesised de novo when a new secretory granule is to be made? 2. Following exocytosis is the secretory granule membrane degraded in lysosomes or is it re-used? Initially labelling studies suggested that secretory and integral membrane proteins were turned over at the same rate. Secretory and membrane proteins of the rat parotid gland, for example, were apparently labelled to the same degree (Amsterdam et al., 1971). However, in many of these early experiments membrane fractions were heavily contaminated with adhering secretory proteins (Farquhar and Palade, 1981). Similar studies on the biogenesis of chromaffin granule proteins suggested that there was not concomitant synthesis of membrane and secretory proteins (Winkler et al., 1971,1972). This may well have been because, in relation to secretory granule fractions from other tissues, chromaffin granule membranes were isolated with comparatively little contamination from soluble proteins.

In these studies the turnover of membrane proteins was investigated by pulse-labelling medullary cells, during retrograde perfusion of bovine or rat adrenal glands with radiolabelled leucine (Winkler et al., 1971,1972; Gagnon et al., 1976). The major chromaffin cell secretory protein, chromogranin A, was synthesised in the RER then, as shown for the mouse adrenal, transported to the trans region of the Golgi complex where radiolabel was detected in immature secretory granules (Coupland and Kobayashi, 1976). Chromogranin A therefore appeared to take the RER-Golgi route taken

by many other secretory proteins in other cells (Palade, 1975).

The proteins of the membrane which packaged these newly labelled secretory products were not labelled to any significant degree (Winkler et al., 1971; Gagnon et al., 1976). It was significant that lipids of the membrane in which the secretory products were packaged had not been labelled with [$^{32}$P]-phosphate, whereas microsomal membrane lipids were labelled (Winkler et al., 1972). Similar results were also reported for pancreatic cells (Gerber et al., 1973). Experimental evidence from both the turnover rates of membrane proteins and from electron-dense markers used to follow the internalisation and intracellular routing of secretory granule membrane, suggest that these membranes may be reutilised. The release of secretory products appears to be from the more mature population of secretory granules of adrenal and parotid glands (Sharoni et al., 1976); new granules remaining near the Golgi complex for several hours (Coupland and Kobayashi, 1976; Castle et al., 1972). This sojourn allows the granule to accumulate small molecules and enables its secretory proteins to mature (for example, post-translational proteolytic processing).

## Endocytosis and Membrane Recycling

The major event in the life cycle of the chromaffin granule is the discharge of its contents, secretory proteins and catecholamines, from the cell. This event is triggered by the binding of acetylcholine to receptors on the cell surface. This precipitates a cascade of biochemical steps beginning with membrane depolarisation and an influx of free calcium which triggers the release of the granule contents to the cell exterior. Calmodulin has been implicated as the intracellular calcium receptor (Geisow

and Burgoyne, 1983; Burgoyne, 1984). It is not known how the secretory granule docks with the plasma membrane, but this may involve special calcium-sensitive fusion proteins (Pollard et al., 1973) and a proteolytic event, in which a metallo-endopeptidase has been implicated (Mundy and Strittmatter, 1985).

Following exocytosis the secretory granule membrane must be retrieved from the plasma membrane by endocytosis. This internalisation event prevents a permanent enlargement of the plasma membrane and allows the cell to re-use the secretory granule membrane. The cycle of exocytosis and endocytosis poses many questions about membrane interactions. Does the inserted chromaffin granule membrane retain its integrity? If so, can the chromaffin granule membrane be visualised on the cell surface after exocytosis? How rapid and efficient a process is retrieval? And finally, what is the final intracellular destination of membrane following its recovery?

To study the insertion of a secretory granule membrane and its retrieval requires a biochemical marker for these events. If a protein which is on the inner surface of the secretory granule is used as a marker it will eventually become exposed on the surface of the cell following exocytosis. The biochemical composition and the topology of the chromaffin granule membrane has been extensively studied (Winkler, 1976; Winkler and Carmichiel, 1982). These studies have shown that the carbohydrate moieties of chromaffin granule membrane glycoproteins are located exclusively on its inner (matrix) surface, and are therefore ideally suited as markers for observing exocytosis and following endocytosis (Huber et al., 1979; Patzak and Winkler, 1986). The first question to be asked was; are these glycoproteins exposed at the cell surface following

stimulation with secretagogues? Using antisera to the membrane glycoproteins, dopamine $\beta$-hydroxylase and another known as glycoprotein III, these antigens were identified by immunofluorescence on the cell surface in the presence of secretagogues (Wildman et al., 1981; Phillips et al., 1983; Dowd et al., 1983; Patzak et al., 1984). When the stimulus was removed the antigens disappeared from the cell surface. The removal of the antigens appeared to be a specific process, in that, the retrieval of dopamine $\beta$-hydroxylase and glycoprotein III was a metabolically dependent, active process (Patzak et al., 1984) blocked by sodium azide (Raff and De Petris, 1973), while the lateral movement of these antigens within the plane of the membrane was not affected (Patzak et al., 1984).

The fusion/fission events in both exocytosis and endocytosis require a continuity to be established between the lipid bilayers of the chromaffin granule membrane and the plasma membrane of the adrenal cell. These membranes have fluid lipid bilayers and a corollary of this fluidity is rapid diffusion of both lipid and integral membrane proteins, and hence a randomisation of components within the plane of the membrane (Schlessinger et al., 1977). So how is the integrity of the two interacting membranes retained?

Morphological evidence suggests retention of membrane domains with the complete removal of patches of inserted membrane, as proposed by Palade (1959), rather than any dispersion of the inserted membrane components within the plasma membrane (Nagasawa et al., 1971; Nagasawa and Douglas, 1972). Cells create differentiated regions within their plasma membranes during secretion, by using the stabilising interactions created by the interaction of integral membrane and cytoskelatal proteins, such as actin (Palade, 1975).

After secretion patches of secretory granule membrane are retrieved by adsorptive endocytosis. During this process the chromaffin granule membrane is segregated into "coated-pits" (Nagasawa and Douglas, 1972). The coating protein ($M_r$180,000) named clathrin (Pearse, 1976) constructs a geodetic lattice (Unanue et al., 1981) around the membrane to be endocytosed and thus appears to act as a molecular filter, excluding other proteins of the plasma membrane. Once the coated pit has budded into the cytoplasm, as a coated-vesicle, the clathrin coat is shed to leave an endocytotic vesicle. Internalisation is a rapid process, with a half time for removal of eighteen minutes, as shown for parotid acinar cells following their stimulation with isoproterenol (Koike and Meldolesi, 1981). In chromaffin cells, granule membrane proteins were all removed from the plasma membrane thirty minutes after stimulation (Lingg et al., 1983; Phillips et al., 1983; Patzak et al., 1984).

**Reutilisation of Secretory Granule Membrane.**

**1. The Evidence for Degradation.**

Palade (1959) proposed that internalised secretory granule membranes were returned to the Golgi complex to be reused in the packaging of new secretory products, an idea supported by experiments with pancreatic acinar cells (Meldolesi and Cova, 1971; Meldolesi, 1974a). However, in contrast to the biosythetic studies described above, the bulk of the morphological evidence from chromaffin and other cells suggested that the markers used for tracking the intracellular route taken by endocytosed material was delivered to lysosomes for degradation (Masur et al., 1972; Geuze

and Kramer, 1974; Kalina and Robinovitch, 1975).

When the membrane retrieval process in rabbit adrenal medullary cells was overloaded by subjecting the animals to insulin shock, there appeared to be a correlation between the disappearence of their electron-dense secretory granules and the appearance of transluscent microvesicles in the cytoplasm on electron micrographs (Koerker et al., 1974). The use of electron dense tracers for microscopy, such as horse radish peroxidase (Abrahams and Holtzman, 1973) and thorium dioxide (Nagasawa and Douglas, 1972; Suchard et al., 1981) suggested that following internalisation, these soluble markers were routed to lysosomes within coated vesicles. Such morphological data, suggesting degradation in the lysosomes (Holtzman et al., 1978), appeared to correlate with early turnover data from many cells which had indicated a concomitant synthesis of both membrane and secretory proteins. However, this data was not consistent with that from studies with chromaffin cells and in addition it had been shown that the cells of the adenohypophysis transferred horse radish peroxidase to the Golgi region and into newly formed secretory granules (Pelletier, 1973).

It is now recognised that many of the morphological markers are content rather than membrane markers, and they themselves may potentiate the removal of endocytosed material to lysosomes. Therefore the choice of a marker for intracellular routing of endocytosed material must be made with care.

## 2. The Evidence for Reutilisation.

Dextran, which in contrast to the soluble protein tracers is relatively inert, has been used to follow endocytosis in isolated acini of rat parotid or lacrimal glands (Herzog and Farquhar, 1977).

In addition to being transported to lysosomes, this marker was concentrated in the dilated rims of the Golgi complex and in condensing vacuoles. Cationised ferritin binds avidly to membranes and has been used as an endocytotic marker in rat anterior pituitary cells (Farquhar, 1978) and in plasma cells (Ottosen et al., 1980). This marker was internalised in coated vesicles then delivered to endosomes, lysosomes, the Golgi complex and to newly formed secretory granules. Endosomes appeared to fuse mainly with the trans-most cisternae of the Golgi complex, from which new secretory granules bud. These experiments suggested that much of the endocytotic traffic was recycled to package new secretory products (Farquhar, 1982).

Such experiments did not indicate which of the two possible routes; plasma membrane to Golgi, or plasma membrane to lysosomes, was the major one in vivo. Experiments in which myeloma cell plasma membrane proteins were radioiodinated to follow internalisation (Wilson et al., 1981; Farquhar, 1982) have shown that the route for endocytosed membrane is predominantly plasma membrane to Golgi complex; this was the only organelle in which a significant amount of radiolabel was concentrated. The few lysosomes present were not significantly labelled. These experiments are supported by the demonstration that the transferrin receptor, tagged with antibody, is recycled to all the cisternae of the Golgi complex (Woods et al., 1986). Cationized ferritin, like horse radish peroxidase, may encourage the cell to manufacture more lysosomes (Farquhar, 1982), although Holtzman (1977) has thought this unlikely. Most of the studies with tracers such as HRP and cationised ferritin have been carried out under conditions in which cells were stimulated to secrete at abnormally high rates (see Meldolesi, 1974a). In such

cases where secretion outstrips retrieval of granule membrane, the plasma membrane may become deeply invaginated (Meldolesi and Ceccarelli, 1981) as the recycling pathway is overwhelmed. So, to maintain the integrity of its plasma membrane, a cell may be forced to open up the degradative route to the lysosomes, to cope with the endocytosed material.

Thus, this traffic to the Golgi complex suggests that the granule membrane is reutilised for packaging new secretory products. However, this does remain to be demonstrated formally in the case of the chromaffin cell. These results, in conjunction with data that suggest that the membrane proteins of the exocrine pancreas, parotid and adrenal medullary secretory granules all turn over at a lower rate than their soluble secreted proteins (Meldolesi, 1974b; Castle et al., 1975; Wallach and Lin, 1973; Winkler et al., 1974; Winkler, 1977), leave us little reason to doubt that following secretion under normal conditions, secretory granule membranes are reused to package newly synthesised proteins in the region of the trans cisternae of the Golgi complex.

**The Maturation of Chromaffin Granules.**

**1. The Chromaffin Granule Matrix.**

As chromaffin granules mature two major events take place, the concentration of their protein content and accumulation of small molecules. The chromaffin granule membrane is relatively impermeable to ions, however, it is able to take up from the cytoplasm and store high concentrations of many small molecules such as catecholamines and nucleotides (Table 1.2). Despite the appearance of hyperosmotic conditions (see Table 1.2), the osmolarity within the granule matrix is equivalent to that of the

Table 1.2. Contents of the Chromaffin Granule Matrix.

| Component. | Concentration. |
|---|---|
| Catecholamines | 550mM |
| Total Nucleotides | 220mM |
| ATP | 160mM |
| Calcium | 17mM |
| Magnesium | 5mM |
| Ascorbate | 22mM |
| Soluble Protein | 200mg/ml |
| pH | 5.7 |

These values are typical of those reported for the main chromaffin Granule matrix components. Data has been taken from Njus *et al.* (1981) and Phillips and Apps (1979).

cytoplasm. The granule matrix is a non-ideal solution, intragranular components interacting to reduce osmotic effects; charge interaction between catecholamines and ATP is an important feature of this (Kopell and Westhead, 1982). Nuclear magnetic resonance spectroscopy has suggested that tightly-bound complexes of high molecular mass do not form at the low pH (5.7) of the granule matrix and that the matrix is fluid with molecules tumbling rapidly (Daniels et al., 1974; Sharp and Sen, 1978).

The mechanism for the concentration of the acidic proteins within the chromaffin granule (to about 200mg/ml) is not known. It has been proposed that calcium may serve to 'aggregate' proteins in the early steps of granule formation and thus play a role in their concentration (Wallach and Schramm, 1971; Amsterdam and Jamieson, 1974; Palade, 1975). Calcium appears to be preferentially accumulated by immature chromaffin granules prior to catecholamines and ATP accumulation (Pletscher et al., 1974; Ceccarelli et al., 1975). Because secretory granule membranes show a low degree of permeability to small molecules, active transport mechanisms must exist.

2. Accumulation of Catecholamines.

Biosynthesis of catecholamines occurs in the cytoplasm (Kirshner, 1975) and they are accumulated by maturing chromaffin granules. The mechanism of catecholamine uptake is well understood (reviewed by Njus et al., 1981). It is an energy dependent process driven by the hydrolysis of ATP. A cytoplasmically oriented $Mg^{2+}$-dependent ATPase powers the translocation of protons into the granule. The granule utilises the electrochemical gradient that it has established by this translocation to accumulate catecholamines, coupling the efflux of two protons to the uptake of one

catecholamine molecule (Njus and Radda, 1978; Johnson and Scarpa, 1979; Apps et al., 1980c; Kanner et al., 1980; Knoth et al., 1980).

3. Accumulation of ATP.

Chromaffin granules maintain high concentrations of ATP and other nucleotides (Table 1.2). However, these are not synthesised by chromaffin granules (Peer et al., 1976) but are accumulated by an energy-dependent process. The membrane potential established as a consequence of proton translocation, rather than the proton gradient itself (Weber and Winkler, 1981; Aberer et al., 1978) appears to be responsible for the accumulation of $ATP^{4-}$ (Weber and Winkler, 1981). However, experiments with resealed 'ghosts' have failed to support this simple idea (Grueninger et al., 1983).

4. Accumulation of Calcium Ions.

Chromaffin granules maintain high concentrations of calcium (Table 1.2) (Phillips et al., 1977). Accumulation of calcium ions in mature granules may be a "scavenging" activity for maintaining low cytosolic free calcium ion levels; released later during exocytosis (Niedermier and Burger, 1981; Kanagasuntherm and Lim, 1981). In many other cells calcium ions are removed to an intracellular compartment such as the mitochondrion or the endoplasmic reticulum.

The mechanism of calcium ion uptake is unknown and has been difficult to study in chromaffin granule preparations due to leakage of intragranular calcium ions and the presence of contaminating mitochondria. As the chromaffin granule membrane is not freely permeable to calcium ions, a carrier mediated transport process has been implicated (Johnson and Scarpa, 1976). This transport may be ATP dependent (Häusler et al., 1981) however experiments with chromaffin granule 'ghosts' (Phillips, 1981) have implicated instead a $Na^{+}$-$Ca^{2+}$ exchange mechanism similar to that characterised for

heart mitochondria (Crompton et al., 1977). This was subsequently shown to be the case for intact granules (Krieger-Brauer and Gratzl, 1982).

## The Chromaffin Granule Membrane.

In molecular terms relatively little is known about the majority of the endomembrane system. Of its many compartments the secretory granules, and in particular the chromaffin granule, are the best characterised component. These studies have been successful in the molecular characterisation and establishing the topography of granule membrane components, but there has been only limited success in relating molecular structure to function.

### 1. Lipid Composition.

Secretory granule membranes have a lipid composition which closely resembles that of the plasma membrane. Both membranes have high lipid to protein ratios, being particularly enriched in sphingomyelin and cholesterol (Winkler, 1976 and 1977). The chromaffin granule membrane itself is unusual in having high levels of lysolecithin (Da Prada et al., 1972). This may be due to the post-mortem hydrolysis of lecithin (Arthur and Sheltawy, 1980); however, the rapid isolation of membranes from human pheochromocytomas has suggested that this is not the case (De Oliveiria-Filgueiras et al., 1981). It has been postulated that lysolecithin may play a role in fusion between membranes (Howell and Lucy, 1969). However only 10% of this phospholipid is in the outer leaflet of the lipid bilayer in chromaffin granule membranes (De Oliveiria-Filgueiras, 1979).

2. Proteins.

A feature which distinguishes secretory granule membranes from the remainder of the endomembrane system is their relatively simple protein composition. In fact much of the membrane protein content of the chromaffin granule and pancreatic and parotid gland secretory granules appears to be unique (Meldolesi et al., 1978). A number of enzyme activities have been unequivocally associated with the chromaffin granule membrane: dopamine $\beta$-hydroxylase, phosphatidylinositol kinase, a proton-translocating ATPase (ATPase I) and a b-type cytochrome. These enzymes reflect the role these granules play in storing small molecules in addition to secretory proteins.

a. Dopmine $\beta$-hydroxylase.

(i) Catalysis.

The biosynthesis of catecholamines up to dopamine is located exclusively in the cytoplasm; the enzymes involved being translated on free polysomes (Sabban and Goldstein, 1984). Dopamine must be actively transported into the chromaffin granule for its hydroxylation catalysed by dopamine $\beta$-hydroxylase, situated on the inner surface of the granule membrane (Kirshner, 1962). The majority of this enzyme is synthesised on membrane bound polysomes (Sabban and Goldstein, 1984). To be converted to adrenaline, noradrenaline must return to the cytoplasm for methylation; the mechanism of this efflux is not known. Adrenaline is finally transported back into the chromaffin granule for storage. Dopamine $\beta$-hydroxylase is a mixed function oxidase which catalyses the splitting of an oxygen molecule so that one atom is donated to noradrenaline while the other gets reduced to water. The enzyme has four subunits each containing two moles of copper to act as

intermediate electron carriers (Ash et al., 1984; Klinman et al., 1984). In vitro the enzyme will accept electrons from a number of co-reductants. However, in vivo two molecules of ascorbate each donate an electron to the enzyme and in the process are oxidised to semidehydroascorbate (Skotland and Ljones, 1980; Diliberto and Allen, 1981). Although the chromaffin granule maintains a high concentration of ascorbate (Table 1.2) the granule membrane is impermeable to this reductant (Ingebretsen et al., 1980). However, the granule appears to get out of this impasse by transporting electrons across the membrane through the transmembrane cytochrome.

(ii) Structure.

Dopamine $\beta$-hydroxylase is the major glycoprotein of the chromaffin granule membrane (Hörtnagl et al., 1972) accounting for up to 25% of the protein associated with membrane preparations (Winkler, 1976). A tetrameric protein, $M_r$300,000, it can be dissociated by guanadinium hydrochloride into dimers of $M_r$150,000. Reduction with thiol reagents produces monomers with $M_r$ between 70,000 and 75,000 (Ljones et al., 1976; Skotland et al., 1977; Saxena and Fleming, 1983). DBH is present in two forms; as a membrane-bound species and a minor soluble species. When the subunits of these two forms are analysed by polyacrylamide gel electrophoresis and staining with Coomassie Blue, or by immunoblotting or lectin overlaying, they appear to be identical (Saxena and Fleming, 1983; Gavine et al., 1984; Apps et al., 1985). The subunits of the two forms of DBH do not appear to be electrophoretically identical, however, it has not been possible to determine whether this is due to differences in the polypeptide chain or whether it is solely due to the heterogeneity imparted by their carbohydrate content.

Oligosaccharides account for 4% of the relative molecular mass of DBH, composed of the sugars mannose, galactose, fucose, N-acetyl glucosamine and sialic acids (Fisher et al., 1981; Fischer-Colbrie, et al., 1982; Gavine et al., 1984; Margolis et al., 1984). This complement of sugars, the proteins lectin-binding properties and its sensitivity to tunicamycin during biosynthesis suggests that its sugars are N-glycosidically linked to the protein. On average DBH contains four biantennary and two high mannose type oligosaccharides, there must therefore be at least two types of nonidentical subunits, which differ at least in glycosylation. All the subunits appear to be glycosylated (Margolis et al., 1984)

To obtain more information about the structure of DBH recent investigations have concentrated on the products of both cell-free translation systems and the cellular synthesis of the enzyme by rat pheochromocytoma (PC12) cells. However, the nature of the unglycosylated subunits, which is controversial, will be discussed in Chapter 6.

b. Cytochrome $b_{561}$

Cytochrome $b_{561}$ is the other major protein of the chromaffin granule membrane (Hörtnagl et al., 1971); if a relative molecular mass of 28,000 is assumed, it may account for 17-20% of the total membrane protein (Winkler, 1976). Spectroscopic analysis had identified the presence of a haem-containing protein, but it was not until the membrane proteins were analysed by two-dimensional polyacrylamide gel electrophoresis that the polypeptide was identified (Apps et al., 1980a). This analysis identified a protein which stained for haem after non-denaturing first dimension isoelectric focusing analysis. After purification it focused as a

single spot with a pI of 6.2. Cytochrome $b_{561}$ is present in the membrane at 6-7nmol of haem/mg of protein (Pollard et al., 1973; Terland and Flatmark, 1980) and is the main component of a Coomassie Blue staining band which on one-dimensional electrophoretograms was previously known as chromomembrin B (Hortnagl et al., 1971).

Cytochrome $b_{561}$ is an integral membrane protein and has been purified following its solubilisation with nonionic detergents (Hörtnagl et al., 1971; Silsand and Flatmark, 1974; Apps et al., 1980a; Flatmark and Grønberg, 1981; Duong and Fleming, 1982). When solubilised and subjected to phase separation in Triton X-114 the cytochrome partitions into the detergent-enriched phase (This thesis; Pryde and Phillips, 1986). However, despite this behaviour the protein has a relatively small complement of hydrophobic amino acids. An amphiphilic protein, it must contain a small membrane-spanning domain with the bulk of the polypeptide apparently exposed at the cytoplasmic surface of the chromaffin granule (Abbs and Phillips, 1980; Huber et al., 1979; Duong and Fleming, 1984). This has been confirmed by proteolysis experiments that have demonstrated the removal of a major antigenic component of the cytochrome from the cytoplasmic surface of the chromaffin granule (Hunter et al., 1982). Relative molecular mass estimations for the cytochrome of between 22,000 and 30,000 have been estimated by electrophoretic analyses (Abbs and Phillips, 1980; Apps et al., 1980a; Duong and Fleming, 1982; Apps et al., 1984). Analytical ultracentrifugation in the presence of Triton X-100 suggests an $M_r$ of 20,500 (Flatmark and Gronberg, 1981).

The function of cytochrome $b_{561}$ as a transmembrane electron translocator and its relationship with DBH, to which it appears to donate electrons, is still controversial. Electron transport from

the cytoplasm to the granule interior appears to be necessary for the reduction of semidehydroascorbate, the product of ascorbate oxidation by DBH (Diliberto and Allen, 1981). The flow of electrons across the chromaffin granule membrane from the cytoplasmic site to its interior can be demonstrated, a positive membrane potential being detected by the accumulation of thiocyanate (Njus et al., 1983; Harnadek et al., 1985). Flow of electrons in the opposite direction has been demonstrated (Grouselle and Phillips, 1982), but that the cytochrome plays a direct role in this transport of electrons and is coupled to reduction of intermediates in the hydroxylation of dopamine remains to be demonstrated.

c. Proton Translocating ATPase.

Chromaffin granules maintain acidic environments within their lumens, as do the Golgi complex, lysosomes and coated vesicles, by hydrolysing cytoplasmic ATP and establishing uncoupler-sensitive transmembrane proton gradients (Bashford et al., 1975a&b; Glickman et al., 1983; Ohkuma and Poole, 1978; Forgac et al., 1983). The electrochemical gradient established across the membrane of the chromaffin granule is used to drive the accumulation of catecholamines and other small molecules (Njus and Radda, 1979; Johnson and Scarpa, 1979; Apps et al., 1980b&c).

The characterisation of the chromaffin granules reserpine-sensitive catecholamine transporter has proved to be difficult and is still controversial (for a recent review see Winkler et al., 1986). The $H^+$-translocating ATPase is, however, relatively well understood. Granule membrane preparations are contaminated by subunits from the mitochondrial $F_1$-ATPase, but these can be removed by washing with sodium bromide (Cidon and Nelson, 1983). These washed membranes have been found to retain two ATPases

(Cidon et al., 1983; Percy et al., 1985). The first, ATPase I, translocates protons and is coupled to catecholamine transport. It has a relative molecular mass of about 400,000 (Apps and Reid, 1977; Apps et al., 1982; Apps et al., 1983) and apparently contains several polypeptide subunits. It is inhibited by N-ethylmaleimide, which labels a subunit of $M_r$ 70-72,000 (Percy and Apps, 1986) and by DCCD which labels a very hydrophobic polypeptide with $M_r$ 7,000 (Sutton and Apps, 1981).

The second ATPase II, which has a relative molecular mass of about 140,000 (Apps et al., 1983), is sensitive to inhibition by orthovanadate. This is characteristic for enzymes of the $E_1E_2$ -type, such as plasma membrane $Na^+,K^+$-ATPase. The role of this second ATPase is quite unknown at present. The two ATPases can be separated after solubilisation and phase partitioning in Triton X-114 (This thesis; Percy et al., 1985; Pryde and Phillips, 1986).

d. Glycoproteins.

The only other major class of chromaffin granule membrane proteins which have been studied are its glycoproteins. Carbohydrate structures on these proteins are restricted to the inner or matrix face of the membrane (Huber et al., 1979; Abbs and Phillips, 1980). Sugar analyses and the lectin binding properties of these proteins have been reported (Huber et al., 1979; Fischer-Colbrie et al., 1984; Gavine et al., 1984). While structural and topological information on these membrane glycoproteins is available their functions are quite unknown.

It will be shown in Chapter 5 that these glycoproteins fall into two operationally distinct families; those that show marked heterogeneity in their isoelectric point and those that show none.

This behaviour may be due in part to the capping of complex oligosaccharides with sialic acid residues. However, it has become clear that other post translational modifications such as phosphorylation and sulphation may be largely responsible for the isoelectric behaviour of these proteins.

# CHAPTER TWO

# MATERIALS AND METHODS

Materials.

1. Chemicals and Biochemicals.

o-Acetylcholine (dried *in vaccuo* with sulphuric acid); acridine orange; ADP; AMP; ATP (vanadate free); ascorbic acid; aprotinin; benzamidine; 4-chloro-1-naphthol; dithiothreitol; ethidium bromide; fumaric acid; glucose-6-phosphate; $\beta$-glycerophosphate; pnp-glycosides; Hepes; p-hydroxymercuribenzoate; iproniazid phosphate; Mes; p-methylaminophenol sulphate; Mops; Nbf-Cl; NP40; quercetin; sodium orthovanadate; Triton X-100; Tween 20; tyramine chloride; UDP-galactose and other general biochemicals were from Sigma Chemical Co. Ltd. Poole, Dorset.

FCCP; NADH; phosphoenolpyruvate and oligomycin were from the Boehringer Corporation (London).

Octaethyleneglycol dodecylether was from the Kouyoh Trading Co., Tokyo, Japan.

Poly(acrylamide), carboxyl modified was from the Aldrich Chemical Co., Gillingham, Dorset.

Triton X-114 was from Fluka, Buchs, Switzerland and was precondensed as described by Bordier (1981).

Turanose and Tween 80, were from Koch Light Laboratories Ltd.

SDS (specially pure); Folin-Ciocalteu reagent; TEMED; acrylamide; N,N'-methylene bis acrylamide and all other general chemicals were of AnalaR grade and from BDH, Poole, Dorset.

Ampholines 3-10 were from Biorad and Ampholines 9-11 were from LKB Instruments Ltd., South Coydon, Surrey.

2. Radiochemicals.

N,N'-Dicyclohexyl[$^{14}$C]carbodiimide was from CEA, Gif-sur-Yvette, France.

[7-$^{14}$C]-tyramine hydrochloride; [$^{3}$H]-acetylcholine chloride and Uridine diphospho-D-[6-$^{3}$H]galactose, ammonium salt were from Amersham International.

[$^{14}$C]formaldehyde was from NEN Research Products, Wedgwood Way, Stevenage, Herts. U.K.

3. Enzymes and Proteins.

Protein A from Staphylococcus aureus and Percoll density marker beads were from Pharmacia Fine Chemicals, Sweden.
Collagenase from Clostridium histolyticum (clostridiopeptidase-A EC 3.4.24.3. NO. 103 578); Lactate dehydrogenase (from rabbit muscle) EC 1.1.1.27; Pyruvate kinase (from rabbit muscle) EC 2.7.1.40 and bovine serum albumin, fraction V were obtained from Boehringer Mannheim, GmbH

Lysozyme (egg white) was from Armour Pharmaceuticals Co. Ltd.

Phosphorylase b; ovalbumin (egg white grade V); carbonic anhydrase (bovine erythrocyte) EC 4.2.1.1; cytochrome c Type III; myoglobin (whale skeletal type II); ovomucoid (trypsin inhibitor type III-O from egg white); trypsin inhibitor (soybean type 1-S); concanavalin A; wheat germ agglutinin and lentil lectin were from Sigma. Horse radish peroxidase conjugates of Pisum sativum agglutinin (HRP-PSA), peanut agglutinin (HRP-PNA) and wheat-germ agglutinin (HRP-WGA) were from Kem-En-Tec, Lemchesveg 11, Dk-2900 Hellemp, Denmark.

Sheep anti-mouse horse radish peroxidase conjugate was from the Scottish Antibody Production Unit.

Schleicher and Schuell Nitrocellulose filters (200mm x 200mm; 0.45um pore size) were from Anderman &Co., Ltd., 145 London Road, Kingston-Upon-Thames, Surrey, KT2 6NH.

4. Cell Culture Materials.

DMEM; F-12 (HAM); RPMI-1640 select-amine kit; Fungizone and nystatin were obtained from Gibco-Ltd, Paisley, Scotland.

New born calf serum, antibiotics and culture plastic ware, were obtained from Flow laboratories, Irvine, Scotland.

Deoxyribonuclease I; neutral red and trypan blue were from Sigma.

5. Other Materials.

a. Toulene fluor.

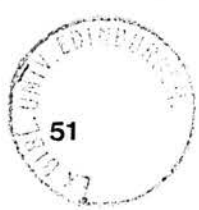
12.5g of 2,5 diphenyloxazole and 0.75g of 1,4,di 2(5-phenoazoyl)benzidine (from Koch-Light) were dissolved in 2.5 litres of AnalaR Toluene.

b. Dialysis tubing.

Visking dialysis tubing was boiled for 2h in distilled water containing EDTA and sodium hydrogen carbonate to bring the pH to 7, followed by a further two boilings in fresh distilled water. The tubing was stored in 50% ethanol at 4$^{o}$C and before use washed with distilled water and the appropriate buffer.

c. Recrystallisation of Digitonin.

Digitonin was dissolved in methanol at 50mg/ml at 65$^{o}$C. After allowing the solution to cool to 45$^{o}$C digitonin was precipitated by the addition of 5vol of diethyl ether. The precipitate was collected by centrifugation at 2000rpm for 10min (20$^{o}$C) in Corex glass tubes. The precipitate was washed three times in



diethyl ether. After drying at 80°C the recrystallised digitonin was broken into a fine powder and stored at 20°C. Solubility - a 20% (w/v) solution of purified digitonin should not precipitate overnight at 4°C.

**d. Periodate Treatment of BSA for Lectin Overlay Blocking.**

BSA (4%,w/v) for lectin overlays was treated with 10mM-$NaIO_4$ dissolved in 0.1M-acetic acid (Glass et al., 1981), for 6hrs at 20°C. Glycerol (10mM) was added and the solution dialysed against 1000vol. of Tris/salt buffer containing 0.5mg/ml $NaN_3$ for 18hr, then diluted to 3% (w/v) BSA.

Subcellular Fractionation of Adrenal Medullary Tissue.

Bovine adrenal glands were placed on ice within 20min of slaughter at a local abbatoir and transported to the laboratory where the medullae were removed within two hours of slaughter. Solutions for tissue preparations were made by dilution of stocks of 2.5M-sucrose and 1M-Hepes (free acid) adjusted to pH 7.4 with 4M-NaOH at 20°C (the final pH after dilution was 7.2). The refractive index of sucrose solutions was measured on a Bauch Lombe refractometer. During preparations all solutions were kept on ice. Centrifugation procedures were at 4°C using Beckman J21B preparative, and Beckman L8 ultracentrifuges.

1. Adrenal Medullary Homogenate.

Following the removal of adhering fat and lateral dissection of the gland, the medulla was freed from the cortex by dissection and placed in buffered-sucrose; 0.3M-sucrose, 20mM-Hepes NaOH, pH7, 0.2mM-PMSF, 1mM-benzamidine. The tissue was passed through a stainless steel mincer, then homogenised by five passes with a motor-driven Teflon pestle in a Potter-Elvehjem homogeniser to give a 25% (w/v) homogenate in a final volume of 500ml.

2. Chromaffin Granule and Mitochondrial Fractions.

The homogenate was cleared of cell debris and nuclei by centrifugation at 4000rpm ($g_{av}$ 1,500) for 5min in a Beckman rotor (type JA14). The substantial pellet was discarded and the post-nuclear supernatant diluted to 420ml with buffered-sucrose before centrifugation at 14,000rpm ($g_{av}$ 19,000) for 30min in the

same rotor.

The pellets, consisting of a pink inner ring of secretory granules and an upper fluffy layer of mitochondria were resuspended by gentle homogenisation in 300ml of buffered-sucrose, then centrifuged at 15,000rpm ($g_{av}$ 18,000) for 20min in a Beckman rotor (type JA20). The pellets were resuspended in 120ml of buffered sucrose and 20ml aliquots layered over 50ml of 1.8M-sucrose, 20mM-Hepes NaOH, pH7.2 in polycarbonate bottles (38x102mm; 70ml) and centrifuged at 45,000 rpm ($g_{av}$ 161,000) for 90min in a Beckman rotor (type 45Ti).

### 3. Chromaffin Granule Membranes.

The pellets ('pure granules') were resuspended by homogenisation in 20mM-Hepes NaOH, pH7.2, 0.2mM-PMSF, 1mM-benzamidine, (referred to below as buffer) using a Teflon pestle, followed by dilution to 210ml with this buffer. The suspension was centrifuged at 45,000rpm for 60min. The resulting supernatant contained the granule matrix components (lysate); the pellets were crude chromaffin granule membranes. The pellets were resuspended by homogenisation in buffer, and 2x20ml aliquots were layered over 50ml of buffered 1M-sucrose (Schneider, 1972). After centrifugation for 60min at 45,000rpm in a Beckman rotor (type 45Ti) purified membranes were collected from the 1M-sucrose interface with a Pasteur pipette; mitochondria sedimented through the sucrose. The purified chromaffin granule membranes were resuspended in 140ml of buffer and centrifuged for 60min at 45,000rpm.

4. Mitochondrial Membranes.

These membranes were recovered from the 1.8M-sucrose interface, lysed and subjected to the same procedure described for the chromaffin granule membrane purification. The mitochondrial lysate was discarded.

5. Chromaffin Granule Lysate.

The lysate (210ml) was supplemented with fresh 0.2mM-PMSF, then dialysed against 1000vol. of 1mM-EDTA, 1mM-Hepes NaOH, pH7.2 for 3 days, lyophilised, then solubilised in distilled water and centrifuged for 2hr at 50,000rpm ($g_{av}$ 171,000) in a Beckman rotor (type 70.1 Ti) before being filtered through nitrocellulose (0.45μm pore size) and stored at -20°C.

6. Microsomal Membranes.

The supernatant from the 14,000rpm centrifugation was adjusted to 410ml with buffered-sucrose and centrifuged at 15,000rpm ($g_{av}$ 18,000) for 20min in a Beckman rotor (type JA20). This ensured removal of residual chromaffin granules and mitochondria. The microsomal fraction was then recovered as a pellet, after centrifugation at 45,000rpm for 60min.

Microsomal fractions were also prepared as described by Bretz and Staubli (1977). The post-granule supernatant was adjusted to 320ml with buffered-sucrose and cleared of residual chromaffin granules as described above. Then 20ml of 1.4M-sucrose, 20mM-Hepes NaOH, pH7.2 was layered under 50ml samples of the supernatant in polycarbonate 45Ti rotor tubes and centrifuged for 60min. The material at the interface was collected. This latter procedure was designed to prevent aggregation of vesicular structures by pelleting

and resuspension. It also enabled residual chromaffin granules and mitochondria to be removed. It was also preferred for protease digestion experiments.

7. Golgi Membrane Enriched Microsomal Fraction.

Microsomal pellets were resuspended by gentle homogenisation in 5-10ml of buffered-sucrose, before being adjusted to 1.4M-sucrose from a 2.5M-buffered-sucrose stock; final volume 45ml. Alternatively, material from 1.4M-sucrose cushion interfaces were readjusted to 1.4M-sucrose. Three step gradients were prepared in polycarbonate 45Ti centrifuge bottles by underlaying with buffered solutions of 15ml of 0.5M-sucrose, 20ml of 0.85M-sucrose, 20ml of 1.15M-sucrose and finally the sample 15ml in 1.4M-sucrose. Gradients were centrifuged at 45,000rpm for 60min in a Beckman rotor (type 45Ti).

Membranes were collected from each sucrose interface (see Figure 3.2), by introducing a glass capillary at the centre of each band of membranes and aspirating. Ten millilitres of the gradient were removed at each interface, diluted with 2vols of buffer, then centrifuged at 45,000rpm for 60min to recover membranes. These were resuspended in a minimum volume of buffered-sucrose and used immediately for assays, or stored at -20$^{o}$C in small aliquots.

Dog Pancreas Microsomes.

Dog pancreas microsomal fractions were prepared by the method described by Walter and Blobel (1983b). They were recovered from the interface of a 1.3M-sucrose/2.5M-sucrose cushion after centrifugation, frozen in liquid nitrogen, then stored at -70$^{o}$C.

Isopycnic Centrifugation on Continuous Gradients of Sucrose.

Sucrose gradients were poured in 13.2ml SW41Ti rotor tubes, the dilute sucrose solution was pumped into the bottom of the tube first. Samples (0.5ml) were either layered on top of the gradients or more generally introduced beneath the gradient from a glass capillary, the sample being chased by 2M-sucrose. Gradients were centrifuged at 40,000rpm ($g_{av}$ 195,000) for 12-15hrs in a Beckman rotor (type SW41Ti) at 4°C. Braking at the end of centrifugation runs was stopped at 3000rpm. Fractions were collected from the bottom of gradients by puncturing the tubes and pumping the gradient samples to a fraction collector.

Washing Membranes with Sodium Carbonate.

Membrane pellets were resuspended in 0.1M-$Na_2CO_3$ (pH11) containing 1mM-EDTA and 0.2mM-PMSF at less than 1mg of protein/ml then left for at least 30min at 0°C (Higgins, 1984; Howell and Palade, 1982). Membranes were recovered by centrifugation at 50,000rpm (227,000$g_{av}$), for 60min in a Beckman rotor (type 50.2Ti), homogenised vigorously and the washing repeated as required. Both the membrane and content fractions were finally dialysed against 360vols. of 1mM-Hepes NaOH, pH7.2 containing 1mM-EDTA. Soluble content proteins were lyophilised, solubilised in water, centrifuged (171,000$g_{av}$ for 60min) and finally filtered through nitrocellulose.

Triton X-114.

1. Solubilisation of Membranes.

Membranes (24mg) were washed in 0.15M-NaCl containing 10mM-Tris HCl, pH7.6 (Tris/salt buffer), centrifuged a 227,000$g_{av}$ for 60min in a Beckman rotor (type 50.2Ti), then resuspended by

homogenisation. Triton X-114 was added from a 10% (w/v) stock at $0^{o}C$ so that final concentrations were 2% (w/v) detergent and 4mg of protein per ml. The solubilised membranes were placed on ice. After one minute a white precipitate formed and 4min later this was removed by centrifugation for 30min at 25,000rpm ($58,000g_{av}$) in a Beckman rotor (type SW50.1). The pellet was washed twice at $0^{o}C$ by resuspension to its original volume, first in the presence of 2% (w/v) Triton X-114, then, after condensation of the detergent, in the absence of detergent. The washed pellet which was subsequently shown to be enriched in cholesterol and phospholipids, was finally resuspended in a minimum volume of Tris/salt buffer (0.5ml).

2. Temperature-induced Phase Separation.

The supernatant left after removal of the precipitate was layered over a 'cushion' of 0.25M-sucrose in Tris/salt buffer (2ml), containing 0.06% (w/v) Triton X-114, in a conical glass centrifuge tube. After 5min at $30^{o}C$ the solution became turbid. It was centrifuged for 5min at 4,000rpm ($2,500g_{av}$) at $20^{o}C$ in a swing-out rotor of a bench centrifuge. A detergent-rich phase was recovered from under the 'cushion' of sucrose.

The aqueous phase above the sucrose was removed and brought to 0.5% (w/v) with Triton X-114, mixed well and placed on ice for dissolution of the detergent. This solution was then layered over the sucrose 'cushion' used above, warmed at $30^{o}C$ for 5min, then the tube was centrifuged. The supernatant was removed, placed at $0^{o}C$, made 2% (w/v) in Triton X114, mixed well and then warmed at $30^{o}C$ for 5min. It was centrifuged to remove the condensed detergent, which was discarded.

After washing the centrifuge tube and the top of the sucrose

'cushion' twice with Tris/salt buffer, the 'cushion' was discarded and the viscous detergent-rich phase was diluted to its original volume with ice-cold Tris/salt buffer to dissolve the detergent. After incubation at 30°C and the detergent phase collected by centrifugation over 0.25M-sucrose again, then diluted with two volumes of Tris/salt buffer and stored at -20°C.

3. Dialysis of the Aqueous Phase.

Residual Triton X-114 was removed from the aqueous phase by dialysis at 4°C against Tris/salt buffer containing 1% (w/v) Amberlite XAD-2. After 1-2 days the medium was replaced and dialysis continued for a further 4-5 days. The dialysed fraction was then diluted with three volumes of water and centrifuged for 2hr at 50,000rpm (171,000$g_{av}$) in a Beckman rotor (type 70.1Ti). The pellet was recovered and washed while the supernatant was filtered through 0.45µm cellulose nitrate.

Labelling Membranes with Dicyclohexyl[$^{14}$C]carbodiimide.

Chromaffin granule membranes and adrenal medullary mitochondrial membranes (4mg of protein/ml) were covalently labelled with dicyclohexyl[$^{14}$C]carbodiimide by incubation in: 0.5mM-EDTA, 10mM-ATP, 20µM-N,N'-dicyclohexyl[$^{14}$C]carbodiimide (specific radioactivity 1.85 GBq/mol) and 10mM-Hepes NaOH, pH7.4. Samples were rotated slowly at 4°C for 15 hrs, then washed in 10 volumes of Tris/salt buffer before being prepared for electrophoresis.

Antisera.

1. Cytochrome $b_{561}$.

Cytochrome $b_{561}$ was purified as described by Apps et al. (1980a), then further purified by electrophoresis on 12% polyacrylamide gels. The Coomassie-stained band of cytochrome $M_r$26,000 to 28,000 was electroeluted then dialysed against 20mM-Tris HCl, pH8, 0.03M-NaCl, 1mM-PMSF before lyophilisation. After solution in water the antigen was emulsified with complete Freunds adjuvant in a Sorval Omni-mixer with a micro-attachment(230). Two young female New Zealand-White Rabbits were used for the immunisation. An initial intramuscular injection at four sites with a total of 200µg of protein was followed six weeks later by an intramuscular booster with 50µg of protein emulsified with incomplete Freunds adjuvant. On the 10th and 14th day following the booster the animals were bled. Blood (25-50ml) was allowed to clot at 20°C for 2hr then left at 4°C for 18hr to allow the clot to contract. The serum was centrifuged at 4,000rpm in a Beckman rotor (JA21) for 20min, then dialysed for 18hr against 20mM-Tris HCl, pH7.6; 0.15M-NaCl, then filtered through nitrocellulose and stored at -70°C in small aliquotes.

2. Chromogranin A

Chromogranin A was purified from granule lysates. After adsorbtion to and elution from DEAE-cellulose (Hogue-Angeletti, 1977) mannose containing glycoproteins were removed by affinity chromatography on concanavalin A-Sepharose. The chromogranin A family of polypeptides were separated under non-reducing conditions on 10% polyacrylamide gels and chromogranin A ($M_r$70,000) was electroeluted from slices of gel and used to immunise rabbits as described above for cytochrome $b_{561}$.

3. Dopamine $\beta$-Hydroxylase.

Soluble DBH was purified from chromaffin granule lysates by ion exchange and lectin chromatography (Ljones et al., 1976) and following preparative electrophoresis in the absence of thiols the $M_r$150,000 DBH dimer was electroeluted from the Coomassie-stained gel and prepared for immunisation as described for cytochrome $b_{561}$.

Electrophoretic Methods.

1. Polyacrylamide Slab Gel Electrophoresis.

Proteins were analysed on 7-15 or 8-15% exponential gradients of acrylamide in the presence of SDS (Douglas and Butow, 1973). The discontinuous buffer system introduced by Laemmli and Favre (1973) was used.

a. Separating Gel.

Gels were poured with a Gilson peristaltic pump into a slab gel cassette (20cmx16cmx1mm) at a flow rate of 1.5ml/min. The gel mix contained: 0.375M-Tris HCl, pH8.8; 2mM-EDTA, 0.1% (w/v) SDS and 0.5% (w/v) polyacrylamide $M_r$250,000, added to increase the mechanical strength of the gel, and acrylamide- N,N bis-acrylamide (stock 30%:0/8%,w/v). The gel was chemically polymerised with 0.05% (v/v) TEMED and 0.05% (w/v) ammonium persulphate. To ensure a flat top, the separating gel was overlayed with water-saturated butan-2-ol. This was washed from the gel once polymerisation was complete.

A 1-2cm stacking gel containing 4.5% (w/v) acrylamide, 0.12% (w/v) N,N' bis-methylene acrylamide and 0.125M-Tris HCl, pH6.8, 2mM-EDTA, 0.1% (w/v)-SDS and 0.5% (w/v)-polyacrylamide was put onto the separating gel and polymerised as described above.

The electrode buffer contained 0.38M-glycine, 0.002M-EDTA,

0.1% (w/v)-SDS, 0.05M-Tris HCl, pH8.3. Gels were run at constant voltage (50-70 volts) at 20°C for 16-20hrs.

b. Sample Preparation.

Before electrophoresis chromaffin granule membranes and samples derived from them were precipitated in 10-75vols. of acetone/ethanol (1:1). These samples were left in Corex glass tubes for at least 60min. on an ice/salt mixture (-18°C) before the precipitated protein was recovered by centrifugation at 15,000rpm in a Beckman rotor (type JA20) for 20min. Pellets were carefully washed with cold water to remove residual solvents and precipitated salts. This procedure was not very efficient for precipitating soluble proteins, so either precipitation with 10% TCA followed by washing with cold ethanol, or lyophilisation was used for these samples.

Precipitated protein was solubilised at 2mg protein/ml, in a sample buffer containing: 0.05M-Tris HCl, pH6.8, 0.002M-EDTA, 5% (w/v) SDS, 10% (w/v) glycerol and 0.001% (w/v) bromophenol blue. Mercaptoethanol (0.5%, v/v) was added if reducing conditions were required. Chromaffin granule membrane samples were not generally heated at 100°C, as in the presence of mercaptoethanol aggregation of hydrophobic proteins occurred. Heating in the absence of mercaptoethanol and its addition after cooling prevented protein aggregation.

c. Fixing and Staining Gels.

Proteins were precipitated in the separating gel with 10% (v/v) acetic acid and 20% (v/v) methanol, then stained with 0.25% (w/v)-Coomassie Brilliant Blue R250 dissolved in 50% (v/v) methanol, 7.5% (v/v) acetic acid for 5-10min. Gels were destained in 10% (v/v)-methanol, 7% (v/v) acetic acid at 40°C, polyeurethane

foam was added to adsorb the free dye.

## 2. Two Dimensional Electrophoresis.

Two dimensional electrophoresis was by the method of O'Farrell (1975).

### a. Isoelectric Focusing.

Isoelectric focusing gels (110mm) were cast in glass tubes (130mm x 2mm inside diam.) sealed at one end with Nesco film. The gel mixture contained: 4.5% (w/v) acrylamide, 0.06% (w/v) bisacrylamide, 9.5M-urea, 2% (w/v)-Triton X-100 and 1.8% (v/v) ampholine pH3-10, 0.2% (v/v) ampholine pH9-11. The gel solution was polymerised with ammonium persulphate [0.04% (w/v)] and overlaid with diluted gel mix without acrylamide. Samples were solubilised in the gel mix without acrylamide, and 0.001% (w/v)-bromophenol blue was added. When required mercaptoethanol was included at 0.5% (v/v). Samples, generally 200-300ug of membrane protein in 50-100µl of sample buffer, were loaded at the cathodic (basic) end of the gel, and protected from the cathode solution, 1% (v/v) ethanolamine, by an overlay solution of diluted gel mix minus acrylamide. The anode solution was 0.5% (v/v) orthophosphoric acid.

Gels were focused at 100V for 30min and 200V until the current was less than 0.5mA per tube, then left for 15-20hrs at 400V at 20$^{o}$C. Gels were put into 'soaking buffer'; 3% (w/v) SDS, 0.05M-Tris HCl, pH6.5, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue for 20-30min, before loading onto the second dimension separating gel. Alternatively, following the removal of the soaking buffer, they were frozen at -70$^{o}$C.

The pH-gradient across the focusing gel was measured by

cutting it into 5mm pieces, placing these in 1.5ml microfuge tubes and adding 0.5ml of boiling distilled water. After 4hr at 20°C the pieces of gel were removed and the pH measured with a micro-electrode (Russell, Auchtermuchty, Fife, Scotland). Many basic proteins cannot be analysed since they focus within the sample load. To detect these proteins samples were loaded from the acid end of the gel.

b. Second-Dimension.

Focusing gel rods were attached to the second dimension gel with 1% (w/v)-agarose dissolved in soaking buffer, care being taken to exclude all air bubbles. Fixing and staining of two dimensional gels was as described for one-dimensioal gels, except that they were fixed for at least 3-4hrs at 40°C to ensure removal of ampholines which stain heavily with Coomassie Blue.

3. Fluorography.

After fixing for 30min, polyacrylamide gels were washed briefly with water, then soaked for a further 30min in 200ml Of 1M-sodium salicylate (Chamberlain, 1979). Gels were placed on Whatmann 3MM paper, covered with polythene film, then dried at 80°C for 1-2hrs on a Biorad slab gel drier.

4. Electrophoretic Transfer of Proteins to Nitrocellulose.

After separation on polyacrylamide gels proteins were electrophoretically transferred to nitrocellulose for decoration with antibodies (Towbin et al., 1979) or radioiodinated lectins (Glass et al., 1981).

a. Immunoblotting.

Transfer to nitrocellulose was for 2hr at 0.8A in an

Electroblot apparatus. The transfer buffer contained: 0.02M-$NaHPO_4$, 0.02% (w/v) SDS, 20% (v/v) methanol. The nitrocellulose was blocked by washing for 1-4hr in 20mM-Tris HCl, pH7.2, 0.15M-NaCl (Tris/salt buffer) containing 3%-BSA and 0.5mg/ml $NaN_3$. This buffer was supplemented with 5% (v/v) inactivated (56°C for 30min) horse serum and antiserum (dilution 1:100 to 1:200), then filtered and the nitrocellulose sheets incubated in it for 90min at 20°C. Following four washes over 30min with Tris/salt buffer containing 0.05% (w/v)-NP40, and a final wash in Tris/Salt buffer without detergent, the sheets were decorated with $^{125}$I-protein A (250,000 cpm/ml) in Tris/salt buffer for 60min. The sheets were finally washed over 30min with five changes of Tris/salt buffer containing 0.05% (w/v)-NP40. After drying autoradiography was done at -70°C.

**b. Lectin Overlays.**

For decoration with $^{125}$I-lectins the transfer procedure was as described above for immunoblotting except that replicas were washed with periodate-treated BSA 3% (w/v) in Tris/salt buffer for 4hr. After washing, the nitrocellulose sheets were decorated by incubation for 16-18hr with $^{125}$I-lectin (10KBq/ml). They were then washed for 4-6hr with Tris/salt buffer, the penultimate wash containing 0.1% (w/v) Triton X-100. After drying, autoradiography was for 3-21days at -70°C. Control replicas were incubated with radiolabelled lectin in the presence of the appropriate hapten sugar (100mM).

**c. Peroxidase Detection of Monoclonal Antibodies.**

Monoclonal antibodies were decorated with sheep anti-mouse horse radish peroxidase (SAM-HRP) with 4-chloro-1-naphthol as substrate. Proteins were transferred from gels to nitrocellulose for 6hrs at 0.8A in phosphate buffer as described above for immune

replicas, except that SDS was omitted from the transfer buffer as it denatured epitopes recognised by monoclonals. After transfer the nitrocellulose was blocked by washing for 90min with 0.1% (w/v) Tween 20 in Tris/salt buffer. Sheets were incubated for 18hr with monoclonal antibody (normally 1:100 dilution) in Tris/salt buffer containing 5% (v/v) normal sheep serum and 0.05% $NaN_3$. This solution was filtered before use through 0.45μm nitrocellulose and could be reused many times if stored at -20°C between blots. Sheets were washed four times in 250ml Tris/salt buffer containing 0.05% (w/v) Tween 20 and finally rinsed with Tris/salt buffer. The monoclonal antibody was then decorated by incubation with SAM-HRP second antibody, 0.1% (v/v) in 80ml of Tris/salt buffer containing 0.1% BSA for 3hrs. The sheets were washed again, as described above, and finally developed by incubation in 200ml of stain solution containing 30ml of 4-chloro-1-naphthol (3mg/ml in methanol), Tris/salt buffer and 60μl of 30% (v/v) $H_2O_2$. The reaction was quenched by washing with water. Blots were photographed, while wet, with Kodak Precision line film LP7.

### 5. Lectin Iodination.

Lectins were solubilised in 50mM-sodium phosphate pH7.2 containing 0.15M-NaCl, 10mM of the appropriate hapten sugar and 1mCi/ml $^{125}I$ and radioiodinated by the two-phase chloramine-T procedure of Tejedor and Ballesta (1982). The iodination reaction done at 0°C was stopped with sodium thiosulphite. Low molecular weight reactants were removed by gel filtration on Bio-Gel P-6DG. Final specific activities were $2x10^6$ to $2x10^7$ Bq/mg of protein and used for overlays at a dilution of 1:500.

### 6. [$^{14}$C]-labelled Protein Standards.

Protein stocks were desalted on Bio-Gel P-6DG then [$^{14}$C]-protein standards for electrophoresis were radiolabelled as described by Dottavio-Martin and Ravel (1978).

### 7. Immunoprecipitation.

The immunoprecipitation of proteins was adapted from the methods described by Anderson and Blobel (1983). Chromaffin cells were washed in 0.3M-Sucrose, 10mM-Hepes NaOH, pH7.2, 1mM-PMSF, 1mM-Benzamidine, Trasylol (400U/ml) ('protease cocktail'). They were homogenised in this buffer using a tight fitting 1ml homogeniser with a Teflon pestle, until greater than 90% of the cells were broken; determined by phase contrast microscopy in the presence of trypan blue. Membranes were recovered by centrifugation at 20,000rpm (27,000$g_{av}$) in a 70.1Ti rotor in 1.5ml microfuge tubes. Microsomal pellets, or cells, were solubilised in 100ul of 4% (w/v)-SDS containing the 'protease cocktail' and boiled for 5min. Four volumes of 60mM-Tris HCl, pH7.4, 0.19M-NaCl, 6mM-EDTA, 2.5% (w/v) Triton X-100 containing 'protease cocktail' was added followed by 20ul of antiserum, and left rotating at 4$^{o}$C for 18hrs. Fifty microlitres of a washed (see below) 50% (w/v) Staphylococcus aureus cell stock was added to the immunoprecipitation mix (0.52ml) and incubated for 2hr (rotating) at 20$^{o}$C.

The IgG-coated bacterial cells were recovered by centrifugation at 2,600xg for 4min in a microfuge and washed five times in 50mM-Tris HCl, pH7.4, 0.15M-NaCl, 5mM-EDTA, 0.02% (w/v)-SDS, 0.1% (w/v) Triton X-100, containing 'protease cocktail'. The final wash was replaced by this buffer without detergent, when samples were for isoelectric focusing. For one-dimensional

electrophoresis the IgG-coated bacterial cell pellet was resuspended in 50µl of [x1] electrophoresis sample buffer containing 0.5% (v/v) mercaptoethanol or in [x1] isoelectric focusing sample buffer which contained 9.5M-urea, 2%-Triton X-100 and 0.5% (v/v) mercaptoethanol. Samples were boiled for 3-5min and the cells removed by centrifugation at 12,600xg for 5min.

Formalin fixed and heat treated Staphyolococcus aureus cells (10% w/v) were washed twice in 50mMTris HCl, pH7.4, 0.15M-NaCl, 5mM-EDTA, 0.4% (w/v)-SDS, 2% (w/v) Triton X-100, containing 'protease cocktail'. Cells were resuspended with a Gilson P200 automatic pipette following their centrifugation at 2,600xg in a microfuge. The washed cells were finally resuspended to 50% (w/v) in the wash buffer.

**Analytical Methods.**

**Enzyme Assays.**

**1. Acetylcholinesterase.**

Acetylcholinesterase (EC 3.1.1.7) was assayed by a modification of the radiometric microassay described by Potter (1967) in a medium containing: 1.6mM-[$^{3}$H]acetylcholine 0.3µCi/ml (23Bq/mol) and 0.1M-Hepes NaOH, pH8, in a final volume of 100µl including sample. After 10min at 37$^{o}$C, 100ul of an ice-cold stop mix containing: 1M-chloroacetic acid, 2M-NaCl and 0.5M-NaOH was added, followed by 4ml of a 9:1 mixture of toluene fluor and amyl alcohol. Samples were vortexed for 2min and then centrifuged at 3000rpm for 5min before scintillation counting. Isotope specific activity was estimated and controls were as is described below for the galactosyltransferase assay.

2. Adenosine 5'-Triphosphatase.

ATPase activity was estimated spectrophotometrically, following the enzymatically coupled oxidation of NADH at 340nm (Pullman et al., 1960). The assay medium contained: 2mM-ATP, 10mM-$MgSO_4$, 1mM-phosphoenol pyruvate, 0.2mM-NADH, 50mM-KCl and 50mM-Hepes KOH, pH7.5 to which the coupling enzymes lactate dehydrogenase (EC 1.1.1.27) 5.5 units/ml and pyruvate kinase (EC 2.7.1.40) 4 units/ml were added. The final volume of the medium was 1ml and it was preincubated at 30°C before addition of the enzyme sample (5-50µl).

In calculating the ATPase activity the volume change due to the sample was taken into consideration. Background NADH oxidation was less than 5nmol/min. A molar extinction coefficient of $6.22 \times 10^6$ $cm^2/mol$ was used to calculate the concentration of the remaining NADH. The effects of ATPase inhibitors on the coupling enzymes were monitored by incubation in the absence of ATP; they had no effect on the oxidation of NADH as demonstrated by the addition of 20nmol of ADP.

3. Cytochrome $b_{561}$.

Membrane samples for the estimation of cytochrome $b_{561}$ content by difference spectroscopy (Apps et al., 1980a) were dialysed against 1000vol of 0.5mM-DTT, 1mM-EDTA and 20mM-Hepes NaOH, pH7 for 18hrs.

Samples, assayed in 1mM-EDTA, 20mM-Tris $SO_4$, pH7.6, were reduced with a few crystals of sodium dithionite, while an identical reference sample was fully oxidised with 80µM-potassium ferricyanide. The concentration of cytochrome was calculated using a molar extinction coefficient of $23.3 \times 10^6$ $cm^2/mol$ (Silsand and

Flatmark, 1974).

4. Cytochrome Oxidase.

Cytochrome oxidase (EC 1.9.3.1) activity was measured by following the continuous oxidation of dithionite reduced cytochrome c at 30°C (Mason et al., 1973). To obtain a linear rate of cytochrome oxidation the following protocol was developed. Cytochrome c (62mg of Type III horse heart) was dissolved in 1ml of 50mM-Mes NaOH, pH6.5, 0.1M-KCl, 1mM-EDTA, saturated with $N_2$, reduced with six small crystals of sodium dithionite, then desalted on a column (11mm dia. x 170mm) of Sephadex G25 (Yonetani and Ray, 1965) equilibrated in the buffer described above. The desalted reduced cytochrome was diluted to 80ml with the assay buffer which contained 50mM-Mes NaOH, pH6.5, 1mM-EDTA and 0.5% (w/v) Tween 80 saturated with $N_2$.

The concentration of cytochrome c and its percentage reduction were calculated from a reduced-oxidised difference spectrum (542nm-550nm) by oxidising the reference cuvette with 50μM-potassium ferricyanide and fully reducing the sample cuvette with sodium dithionite; the solution was then diluted to 50μM cytochrome c. Reference samples contained 2mM-KCN from a fresh stock at 0.2M in 1M-Tris HCl, pH8. Cytochrome c activity was calculated using a molar extinction coefficient of 21 $x10^6$ $cm^2/mol$ at 550nm (Massey, 1959).

5. Cytochrome P450.

Cytochrome P450 content of microsomal fractions was estimated by difference spectroscopy in a Pye Unicam SP1800 spectrophotometer. Membrane samples in 2ml of 50mM-Tris HCl, pH7.4 were reduced with a few crystals of sodium dithionite, then split between two semi-micro

glass cuvettes. A base line was drawn then carbon monoxide bubbled gently through the sample cuvette. The specific content of cytochrome P450 was estimated from the absorbance difference between 450nm and 490nm using a molar extinction coefficient of $91 \times 10^6$ $cm^2/mol$ (Omura and Sato, 1964).

### 6. Dopamine β-Hydroxylase.

The activity of dopamine β-hydroxylase was estimated by a modification of the method of Friedman and Kaufman (1965). Samples were incubated for 15min at 37°C in 100µl of medium containing: 50mM-fumarate, 0.5mM-ascorbate, 13.5mg/ml iproniazid* phosphate, 0.1mM-p-hydroxymercuribenzoate 0.9 units/ml-catalase, 2mM-tyramine chloride, 0.5µCi/ml[$^{14}$C]-tyramine and 100mM-Mes NaOH, pH6.5. The reaction was stopped with 100µl of 20% (w/v)-TCA. The cloudy sample was clarified with 50µl of ammonia solution (35% v/v $NH_3$), then 25µl of 2% (w/v) $NaIO_4$ was added. After 4min 300µl of a 10% (w/v) glycerol/4M-HCl solution then 0.5ml of toluene fluor were added with mixing for 30s. Samples were finally centrifuged at 3000rpm for 5min before scintillation counting.

### 7. Galactosyltransferase.

The transfer of galactose from [$^3$H]-UDP-galactose to the glycoprotein acceptor ovomucoid (Bretz and Staubli, 1977) catalysed by galactosyltransferase (EC 2.4.1.38) was assayed for 60min at 37°C in an incubation mixture containing: 50mM-Mops NaOH, pH7, 20mM-$MnCl_2$, 10mM-DTT, 2mM-ATP, 5mg/ml ovomucoid, 0.5% (w/v) $C_{12}E_8$, 0.2µCi of UDP-D-[6-$^3$H]-galactose/ml (specific activity 20µCi/mmol). The final volume was 100µl, including a sample of up to 50µl. The reaction was stopped with 2ml of ice cold 5%-TCA. Precipitated

*isonicotinic acid 2-isopropyl hydrazide

protein was collected on nitrocellulose filters (2.5cm $^2$; 0.45μm pore size) mounted in an Amicon vacuum manifold and washed with 20ml of 5%-TCA. Time zero incorporation was estimated by the immediate addition of samples to TCA. The specific radioactivity of the reaction mixture was estimated by applying a sample directly to nitrocellulose without washing. Filters were dried and placed in 2ml of toluene fluor for scintillation counting.

ATP was included in the assay medium to protect the substrate from pyrophosphatases (Bretz et al., 1980). Ovomucoid although not the physiological substrate, contains 24 N-acetylglucosamine residues (Davis et al., 1971) on three highly branched chains (Montgomery and Wu, 1963). Under these conditions galactose incorporation was linear with both time and protein concentration. Although counting tritium on nitrocellulose filters may invite quenching problems, the counting efficiency was 40% and each sample was subjected to individual quench correction. By assaying each sample at two or more protein concentrations linearity of product formation was monitored.

### 8. Glucose-6-Phosphatase.

Glucose-6-phosphatase (EC 3.1.3.9) was assayed in a medium containing: 20mM-glucose-6-phosphate, 4mM-EDTA, 2mM-KF, 0.1% (w/v) $C_{12}E_8$ and 40mM-sodium cacodylate pH6.5 in a final volume of 0.4ml containing the sample (usually 20μl) of enzyme. The reaction was stopped by the addition of 0.2ml of 20%-TCA and 0.1ml and 0.2ml samples were taken for estimation of inorganic phosphate.

To estimate the contribution from nonspecific phosphatases to inorganic phosphate liberation, control assays were run in parallel in which glucose-6-phosphate was replaced by 10mM

β-glycerophosphate.

9. α-Glucosidase and α-Mannosidase.

Glycosidase activities were assayed in: 0.1M-Mops NaOH, pH7 containing pnp-α-D-glucoside or pnp-α-D-mannoside. Samples in a final volume of 0.5ml were incubated for 60min at 37°C and the reaction stopped with 0.5ml of 0.2M-$Na_2CO_3$. Release of p-nitrophenol was estimated at 410nm using a molar extinction coefficient of 18.6 x$10^6$ $cm^2$/mol. For each sample a blank was prepared in which the enzyme was added after $Na_2CO_3$. Control assays contained pnp-β-D-glucosides. Stocks of pnp-glucoside were filtered through nitrocellulose (0.45μm pore size).

10. 5'-Nucleotidase.

Samples for assay of 5'-Nucleotide phosphohydrolase (EC 3.1.3.5.) (Michell and Hawthorne, 1965) were incubated in a medium containing: 0.1M-KCl, 10mM-$MgCl_2$, 5mM-AMP, 0.1% (w/v) $C_{12}E_8$, 10mM-sodium potassium tartrate and 50mM-Hepes NaOH, pH7.2. The reaction was stopped and samples processed as described for the glucose-6-phosphatase assay. Sodium potassium tartrate was included to inhibit acid phosphatases.

Chemical Determinations.

1. Dye Binding Assay for Protein.

For routine estimation of adrenal medullary proteins, in albumin equivalents, a dye binding microassay giving an absorbance change of 0.25/5μg of BSA and linear between 2-8μg was used; the concentration of BSA was calculated from its absorbance at 280nm

(Bradford, 1976). A [x5] reagent stock containing 25mg of Serva blue G dissolved in 25ml of 96% ethanol, 50ml of 85% orthophosphoric acid and 25ml of water was stored at 4°C.

2. Modified Lowry Protein Assay.

When detergent was present in samples a modification (Hartree, 1972) of the method of Lowry et al. (1951) was used . This was used as published, except that the assay volume was reduced.

3. RNA.

Acid insoluble RNA was determined as described by Blobel and Potter (1968). To 50µl samples of subcellular fractions 950µl of ice cold 0.3M-perchloric acid (PCA) was added. After 60min on ice samples were centrifuged at 2600x$g_{av}$ for 5min at 4°C. Pellets were washed twice with 1ml 0.2M-PCA, then resuspeded in 250µl of water before the addition of 250µl of 0.6M-KOH and incubation for 60min at 37°C. Following this incubation the tubes were placed on ice for 10min before the addition of 1ml 0.6M-PCA. After 15-30min precipitated protein was removed by centrifugation at 12,600x$g_{av}$ for 10min at 4°C in a microfuge. The absorbance of samples at 260nm using $E^{1\%}_{1cm}$=312 (Munro and Fleck, 1966) was used to estimate micrograms of RNA.

4. Inorganic Phosphate.

Inorganic phosphate was assayed spectrophotometrically as described by Le Bel et al. (1978) and modified for assay in 1.5ml microfuge tubes. To enzyme assay supernatants in a final volume of 0.2ml was added 0.6ml of copper acetate reagent pH4 (0.25% $CuSO_4$ and 2.8% sodium acetate in 2M-acetic acid) and 0.1ml of reducing reagent

(2%-p-methyl amino phenol sulphate in 5% sodium sulphite) and following colour development samples were read at 870nm.

5. Phospholipid.

Total phosphorus was estimated, after extraction of samples into chloroform/methanol (1:1) by a modification of methods developed by Bartlett (1959) and Parker and Peterson (1965). Separation by one-dimensional thin layer chromatography was on 20x20cm K5 plates after first eluting the neutral lipids, using solvent systems developed by Dr. Susan Butler, Department of Cardiology, University of Edinburgh Medical School.

6. Cholesterol.

Cholesterol was estimated spectrophotometrically using $H_2O_2$, liberated by the enzymatic oxidation of cholesterol by cholesterol oxidase, to reduce iodide to iodine, as described in Boehringer Mannheims cholesterol-c system. Samples were compared with a cholesterol standard and assayed using a Cobos Bio centrifugal analyser.

7. Triton X-114.

The concentration of Triton X-114 in samples was estimated as described by Garewal (1973). The only departure from the published procedure was that dichloromethane was used to extract the detergent-cobaltothiocyanate complex. Triton X-114 (not pre-condensed) was used to construct a standard curve.

8. Catecholamine Determination.

Catecholamine was estimated by the fluorescence assay described by von Euler and Lishajko (1961).

**Cell Culture Methods.**

**1. Isolation of Chromaffin Cells.**

Bovine adrenal medullary chromaffin cells were isolated by collagenase digestion of glands by the methods described by Fenwick et al. (1978) and Aunis et al. (1980). All procedures were at 37°C, except for Percoll gradient centrifugation at 20°C. $Ca^{2+}$-free Lockes solution, DMEM, F-12 and other media were prepared as described by Wilson and Viveros (1981).

Adrenal glands encased in fat were placed in $Ca^{2+}$-free Lockes at 37°C, 15-20 minutes after slaughter of animals and transported to the laboratory. The glands were freed of their adhering fat, care being taken to leave a small collar on the main adreno-lumber vein. A plastic cannula was inserted 1cm into the gland and secured with surgical thread. Major leakage from secondary veins was stemmed by suturing. To reduce back pressure during perfusion 1mm deep slashes were made through the capsule and into the adrenal cortex. Three glands were perfused with 200ml of $Ca^{2+}$-free Lockes (flow rate 8ml/min) to wash out erythrocytes.

Glands were perfused with 75ml of collagenase solution, with recycling for 30min (flow rate about 5ml/min). The period of perfusion was dependent on the batch of collagenase. Digestion with collagenase was stopped by reperfusing with $Ca^{2+}$-free Lockes solution. The glands at this stage were swollen and puffy. After lateral dissection with scissors, the medulla was separated from the adrenal cortex and removed to the remaining 25ml of fresh collagenase solution. The tissue was minced with scissors and incubated at 37°C for 15min. Cells were separated from undigested tissue by filtration through a 250µm nylon mesh. They were recovered, after dilution to 200ml with $Ca^{2+}$-free Lockes solution,

by centrifugation at 1000rpm ($800g_{av}$) for 10min in a swing out rotor (referred to below as centrifugation). The cloudy supernatants were discarded and the pellets (1-2ml of packed cells) washed in 200ml of $Ca^{2+}$ Lockes solution and recovered by centrifugation. Before purification on Percoll cells were filtered through an 82µm nylon mesh.

2. Purification of Cells on Percoll.

Percoll was diluted 1:9 with [x10] $Ca^{2+}$-free Lockes solution, 38ml of this was added to 42ml of the cell suspension and the mixture centrifuged for 20min at 15,000rpm ($20,000g_{av}$) in a Beckman rotor (type 50.2Ti). The top 10ml of the gradient containing cell debri, non-viable cells and cortical cells was discarded. The diffuse band of chromaffin cells (see Chapter 6) was removed and washed twice, by dilution to 200ml with DMEM. The cells were finally resuspended in 20ml of DMEM and filtered through an 82µm nylon mesh before counting.

3. Cell Viability and Counting.

The number of chromaffin cells was estimated by diluting 50ul of the cell suspension with an equal volume of 0.4% (w/v) Trypan Blue dissolved in 0.15-NaCl, 0.02% (w/v) sodium azide 5mM-Tris HCl, pH7.6; with viability assessed as the number of cells excluding the dye. Chromaffin cell numbers were also estimated by staining with neutral red (0.3 mg/ml in $Ca^{2+}$-free Lockes soluion) (Stuart et al., 1974; Role and Perlman, 1980). Cell viability was additionally monitored by fluorescence microscopy with acridine orange/ethidium bromide solution (each at 1ppm) (Parks et al., 1979).

4. Primary Cell Cultures.

Chromaffin cells were cultured in Petri dishes or in multi-well plates for two days in medium containing: 45% (v/v) of both DMEM and HAMS-F12 and new born calf serum at 10% (v/v) (Bottenstein and Sato, 1979; Wilson and Viveros, 1981). Cells were cultured in the absence of serum from the third day onwards, media being changed every 2-3 days.

5. Radiolabelling Chromaffin Cells.

Before radiolabelling cells were incubated for one hour at $37^{o}C$ in DMEM and $95\%O_2/5\%CO_2$. To deplete cellular methionine pools they were then incubated for 30min in RPMI 1640 medium lacking methionine.

a. $[^{35}S]$-Methionine.

For both labelling, and pulse chase experiments 0.5ml of cells at $4x10^6$ cells/ml were added to an equal volume of RPMI 1640 containing $[^{35}S]$-methionine at 20-100µCi/ml (details are shown in the relevant Figure legends). For chases with cold $[^{32}S]$-methionine, 100µl of a 0.1M methionine stock containing 10mM dithiothreitol was added to the incubation.

Protein synthesis was stopped by chilling on ice and centrifuging cells for 2min at 2,600xg in a microfuge, then solubilising immediately in sample buffer, for electrophoresis. Alternatively samples were frozen in liquid nitrogen then stored at $-70^{o}C$.

b. Determination of $[^{35}S]$-methionine Incorporation into Protein.

The incorporation of methionine was monitored by taking samples to an equal volume of 20% ice cold TCA and recovering the

precipitated protein by centrifugation. Pellets were washed twice with 10% TCA and once with 1ml of cold ethanol. The pellet was then solubilised in 1ml of 0.4% sodium deoxycholate and 0.1M-NaOH and incubated for 60min at 37°C to hydrolyse transfer-RNA. After precipitation with an equal volume of 20% TCA the sample was recovered on GF/C filters, washed with TCA (20ml of 5%) dried and radioactivity counted in toluene fluor.

c. [$^3$H]Acetate.

Aliquots of [$^3$H]Acetate (1.5mCi) in ethanol were dried in a stream of nitrogen. Cells ($10^7$ cells/ml) in DMEM were added and mixed well. Samples for immunoprecipitation and electrophoresis were taken after 2hr and 18hr at 37°C in 95%$O_2$/5%$CO_2$.

6. Inhibitors.

All inhibitors dissolved in solvents were added to cell incubations at below 1% v/v. Controls contained an equivalent amount of the carrier solvent.

a. FCCP.

Carbonyl cyanide p-trifluoromethoxy phenyl hydrozone was added at 1-10μM from a 2mM stock dissolved in ethanol. Cells were preincubated for 30min with this inhibitor of oxidative phosphorylation.

b. Monensin.

Monensin was added at between 0.1-10μM from dilutions of a 100mM stock in ethanol. Cells were preincubated for 30min or 4hr with this ionophore.

c. Tunicamycin.

Tunicamycin was used at 10μg/ml and added from a 10mg/ml stock dissolved in methanol/dimethyl sulphoxide 3:1. Cells were

preincubated with this inhibitor of N-glycosylation for 4hrs.

7. Protease Treatment.

Microsomal fractions for protease treatment were isolated from $14x10^6$cells, homogenised with a tight fitting pestle until greater than 90% of cells present were broken, determine by microscopy in the presence of trypan blue. The post $800g_{av}$ cell supernatant in 2.5ml of buffered-sucrose was fractionated on a gradient of buffered 0.5M-sucrose (2ml) and 2.5M-sucrose (1ml) by centrifugation at 40,000rpm in an SW50.1 rotor for 60min. Membranes were recovered from the 0.5M/2.5M-sucrose interface. Samples (0.5ml) containing the microsomal fraction from $2x10^6$cells were treated with: 10µg-trypsin/ml, 10µg-chymotrypsin/ml or 2µg-pronase/ml the latter containing 0.3mM-$CaCl_2$. Stocks of the proteolytic enzymes were solubilised in 10mM-TrisHCl pH 7.5, frozen in liquid nitrogen and stored at -70°C, at respectively 5-, 5- and 1mg/ml. Digestions in the presence and absence of 1% (w/v) Triton X-100 were for 60min on ice and stopped by adding an equal volume of 20% (w/v) TCA. Precipitates were collected by high speed centrifugation and washed briefly with cold ethanol before being dissolved in 50µl of sample buffer for isoelectric focusing.

# CHAPTER THREE

## ISOLATION OF A GOLGI-ENRICHED MICROSOMAL FRACTION.

## Introduction.

Golgi fractions retaining their characteristic stacked membrane morphology were first isolated from epithelial cells of rat epidiymus. These membranes were enriched in phosphatase activity and reacted strongly with the carbohydrate stain PAS; the significance of this would only be recognised in later investigations. Golgi enriched fractions were subsequently isolated by differential and isopycnic centrifugation from plant cells (Morre and Mollenhauer, 1964), and rat and bovine liver (Morre et al., 1970). Although thiamine pyrophosphatase activity was enriched in these Golgi membrane fractions (Cheetham et al., 1970,1971) it was not uniquely localised to a morphologically identifiable compartment, which precluded its use as a specific marker (De Duve, 1975). Despite the lack of a quantitative criterion with which to monitor purity, the characteristic morphology of the Golgi stack, enhanced by negative staining for electron microscopy (Morre et al., 1970), was used to assay fractions. Differential labelling of the stacks with osmium tetroxide (Friend and Murray, 1965; Fleicher et al., 1969) suggested biochemical differences between them. Radiolabelling studies with sugars also implicated the Golgi complex in the biosythesis of complex carbohydrate structures (Neutra and Leblond, 1966a&b; Lane et al., 1964), and it was the unique ability of Golgi membrane fractions to transfer galactose from its sugar nucleotide precursor to N-acetylglucosamine residues that provided the first biochemical marker for this organelle (Fleischer et al., 1969; Fleischer and Fleischer 1970; Morre et al., 1969). Other glycosyltransferases adding fucose and sialic acid residues to complex oligosaccharides on glycoproteins were soon shown to be uniquely located within the

Golgi complex (Schachter et al., 1970); this biochemical evidence was supported by autoradiographic studies.

The approach to isolating a Golgi-enriched fraction is to some extent tissue dependent. Tissues poor in SER, such as acinar cells of the pancreas, cells of the parotid gland and the chromaffin cells of the adrenal medulla (Trifaró and Duerr, 1976) can be fractionated into a smooth microsomal and an RER fraction. However, when significant amounts of SER are present, in liver for example, preservation of stacked Golgi membranes is desirable (Cheetham et al., 1971) to enable recovery by low speed differential centrifugation procedures (Morre, 1971). Some of the purest Golgi fractions have been isolated from hepatocytes by inducing very low density lipoprotein overloading by ethanol intoxication of rats facilitating flotation of the Golgi complex away from denser microsomal fractions on sucrose gradients (Ehrenreich et al., 1973; Bergeron et al., 1973). Fractionation of rat liver microsomal fractions by isopycnic centrifugation on sucrose gradients produces three fractions enriched in Golgi elements; a heavy cis ($GF_3$) an intermediate ($GF_2$) and a light trans ($GF_1$) fraction, which have been used to monitor the sequential movement of radiolabelled glycoproteins from one Golgi cisterna to the next (Bergeron et al., 1978; Bretz et al., 1980; Ehrenreich et al., 1973). Such Golgi-enriched fractions have been further purified by affinity adsorption of RER membranes with antibodies to NADPH-cytochrome P450 reductase immobilised on polyacrylamide beads (Ito and Palade, 1978).

In the past few years the biogenesis of the major secretory protein of the chromaffin cell, chromogranin A, has been unravelled (discussed in Chapter 6). However, few attempts have been made to

study the biogenesis of granule membrane proteins. We had produced in our laboratory antisera to three chromaffin granule proteins reflecting three topologically distinct compartments:

1. Cytochrome $b_{561}$, a transmembrane protein with a major antigenic site exposed on the cytoplasmic surface (Abbs and Phillips, 1980; Hunter et al., 1982; Duong and Fleming, 1984).
2. Dopamine $\beta$-hydroxylase located on the inner matrix surface of the granule membrane.
3. Chromogranin A the major secretory protein.

There have been no studies undertaken in which the distribution of these major granule proteins throughout the endomembrane system of the chromaffin cell have been compared. Cytochrome $b_{561}$ has however, been detected in microsomal fractions (Hörtnagl, 1976).

Using the sensitive immunoblotting technique introduced by Towbin et al. (1979) I have attempted to identify these granule proteins, and have screened for putative biosynthetic precursors in subcellular fractions of adrenal medullary tissue. The established role of the Golgi complex in the terminal glycosylation of N-linked oligosaccharides was monitored, for DBH in particular, by $^{125}I$-lectin overlays, two-dimensional electrophoretic procedures providing the resolution required for such an approach.

It was hoped that this type of approach would provide answers to the following questions:

1. Are the proteins of the chromaffin granule membrane unique to it? Previous studies have suggested that this may be the case.
2. If Golgi and RER-enriched microsomal fractions do contain chromaffin granule proteins are they present only as artifacts of subcellular fractionation?

3. Are there integral membrane proteins common throughout the endomembrane system? If there are then these may be candidates for fruitful studies of the components responsible for the sorting and targeting of proteins and for maintainence of membrane integrity.

4. Is there a basal rate of synthesis of membrane proteins in chromaffin cells which can in favourable circumstances be detected by radiolabelling cells or immunologically in tissue fractions by "state-of-the-art" electrophoretic techniques? Since cytochrome $b_{561}$ and DBH between them account for the majority of the membrane protein it is not unreasonable that even at very low rates of turnover these proteins in particular may be detectable.

The objective of the present work was the isolation of Golgi- and RER-enriched fractions in a form as free from contamination by secretory granule membranes as possible. This is a difficult task because of the rather similar buoyant densities of all the relevant membranes. The large secretory granule component of adrenal medullary tissue allows granule membranes to be isolated in a highly pure form from isolated intact granules. Subcellular fractions enriched in endoplasmic reticulum (Hörtnagl, 1976) and Golgi membranes (Trifaró and Duerr, 1976) have been described previously, but the characterisation of such fractions is problematical for the following reasons:

1. Microsomal fractions separated by density gradient centrifugation in sucrose contaminate each other.

2. Many of the traditional marker enzymes were characterised for use in the pioneering studies with rat liver fractions and although routinely applied to other tissues, may not be present in significant amounts in these.

3. Marker enzyme activity must be stable under the isolation

conditions and not sensitive to endogenous inhibitors. For practical purposes it should be stable to at least one cycle of freezing and thawing; DBH for example does not meet this criterion.

In this and the following chapter I describe attempts to isolate and characterise the following membrane fractions:

1. Chromaffin granule membranes.
2. Mitochondrial membranes.
3. Plasma membranes.
4. Golgi-enriched microsomal membranes.
5. RER-enriched microsomal membranes.

The basic strategy adopted was preliminary removal of large particulate material as completely as possible following homogenisation, even at the expense of reducing the yield of other membranes. Membranes remaining in the microsomal fraction were then separated on density gradients of sucrose.

I have investigated, and discuss below, some of the marker enzymes used by previous workers, and I have also investigated some new enzyme activities for these fractions. However, because of their limited usefulness I have augmented these studies with electrophoretic analyses, detailed in Chapter 4.

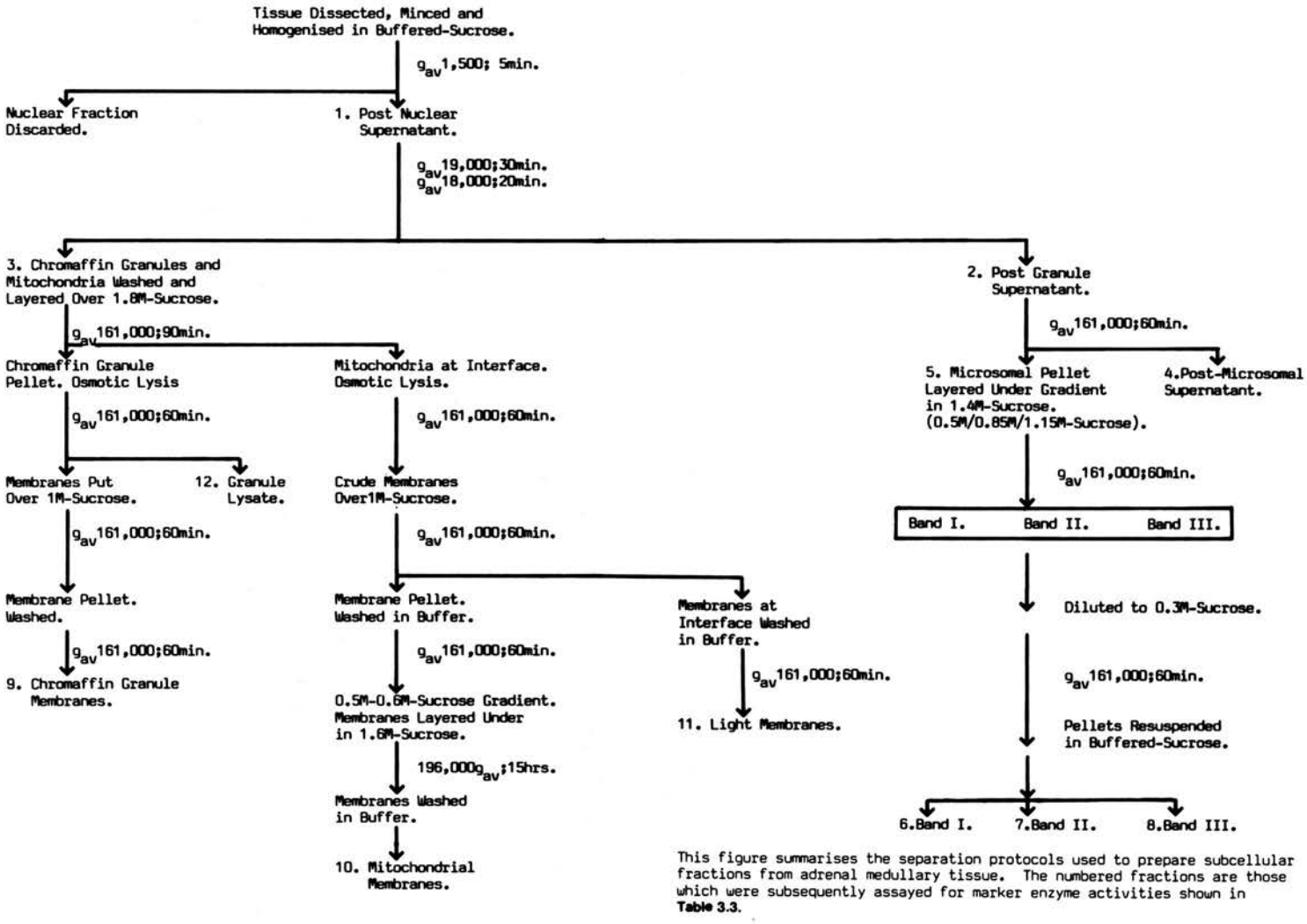
## Fractionation of Microsomal Membranes.

The protocols used to fractionate adrenal medullary tissue are shown in Figure 3.1. These fractions were characterised with enzyme markers which are summarised in Table 3.1.

Microsomal pellets were resuspended in buffered-sucrose, then adjusted to contain 1.4M-sucrose. Initially the microsomal fraction was collected at the interface of a 1.4M-sucrose "cushion". As there was subsequently no difference between the distribution of enzyme markers on sucrose gradients the pelleting procedure was adopted as it enabled cytoplasmic proteins to be washed from the fraction. Membrane fractions were isolated under hypertonic or isotonic conditions to preserve vesicular structures.

A microsomal fraction enriched in the marker for the Golgi complex, galactosyltransferase, was first produced by differential centrifugation. This fraction was then separated on step gradients of sucrose that were designed from the buoyant density data shown in Table 3.2, obtained from preliminary fractionations on continuous gradients of sucrose, examples of which are shown later. In addition, data from the literature was used; in particular from the methods used to isolate Golgi membrane-enriched fractions from rat liver microsomal fractions (e.g. Fleischer and Fleischer, 1970; Bretz and Staubli, 1977). Initially fractions were recovered from four sucrose interfaces and operationally defined as: Band I (0.3M/0.9M), Band II (0.9M/1.1M), Band III (1.1M/1.2M) and Band IV (1.2/1.4M). Band II showed a 10-fold enrichment, relative to the post-nuclear supernatant, in galactosyltransferase and acetylcholinesterase markers for respectively - Golgi membranes and

FIGURE 3.1. Subcellular Fractionation of Adrenal Medullary Tissue.




This figure summarises the separation protocols used to prepare subcellular fractions from adrenal medullary tissue. The numbered fractions are those which were subsequently assayed for marker enzyme activities shown in **Table 3.3.**

Table 3.1. Markers Used to Characterise Adrenal Medullary Tissue Fractions.

| Subcellular Compartment. | Marker. |
|---|---|
| Cortical Membranes | Cytochrome P450. |
| Chromaffin Granule Membranes | Cytochrome $b_{561}$. |
| | Anti-Cytochrome $b_{561}$ Antiserum |
| | Dopamine $\beta$-hydroxylase. |
| | Anti-DBH Antiserum. |
| | Anti-chromogranin A Antiserum. |
| Golgi Membranes. | Galactosyltransferase. |
| | Mannosidase II (pH7.4). |
| Mitochondrial Membranes | Cytochrome Oxidase. |
| Plasma Membranes. | Acetylcholinesterase. |
| | 5'-Nucleotidase. |
| Rough Endoplasmic Reticulum | RNA |
| | Glucose-6-Phosphatase. |
| | $\alpha$-glucosidase. |
| | Anti-Docking Protein Antiserum |

Table 3.2. Buoyant Densities of Adrenal Medullary Membranes on Continuous Gradients of Sucrose.

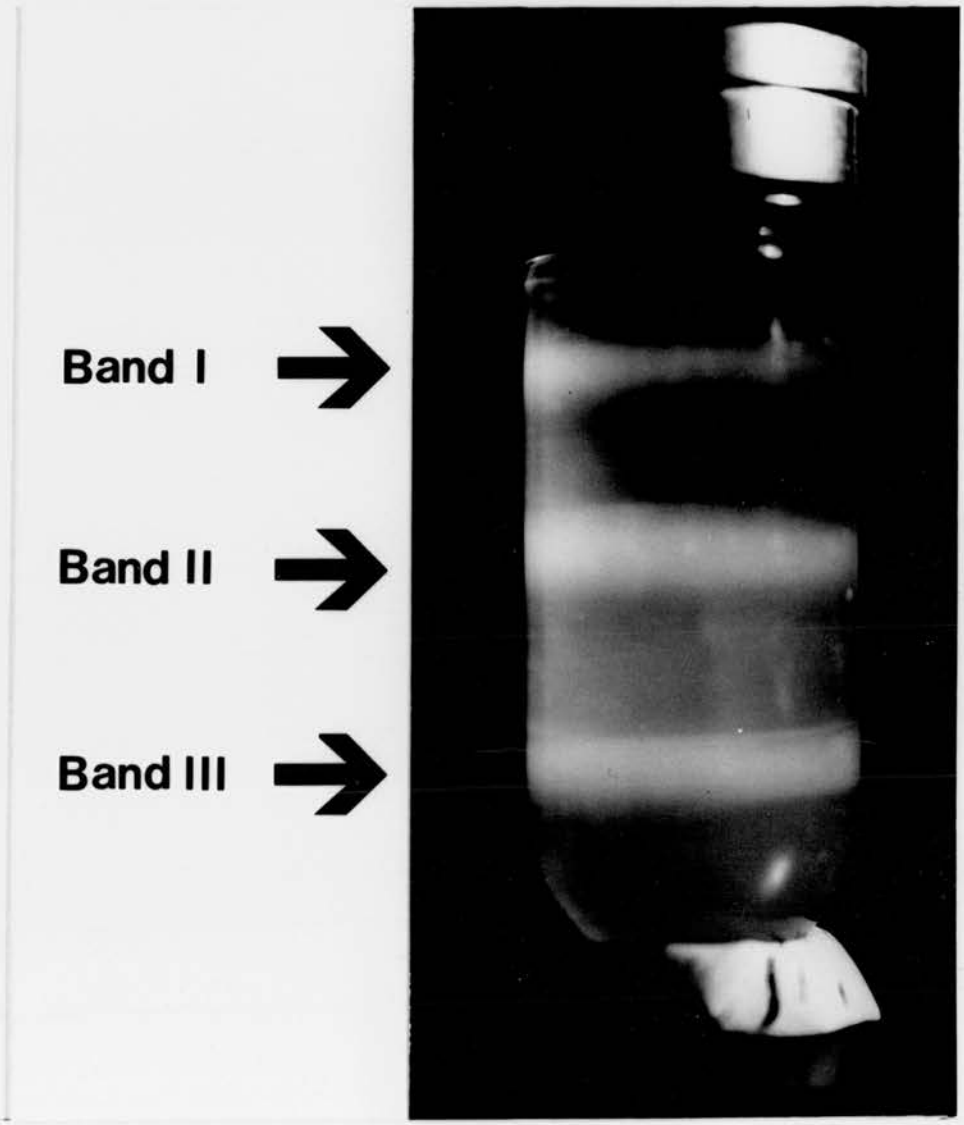
| Membrane Fraction. | Density ($g/cm^3$)*. |
|---|---|
| Plasma. | 1.090. |
| Chromaffin Granule. | 1.100. |
| Golgi. | 1.123. |
| Rough Endoplasmic Reticulum. | 1.175. |
| Mitochondrial. | 1.189. |

Densities were determined by flotation of membranes to equilibrium on 0.5M-1.6M-sucrose gradients. * Density in sucrose equivalents at 20°C.

plasma membranes, suggesting that they were co-sedimenting under these conditions. This step gradient was therefore redesigned to try and increase the enrichment in galactosyltransferase and to remove as much acetylcholinesterase as possible. It was found that the best selective enrichment was obtained under the same conditions used by workers on rat liver (see Table 3.3). Figure 3.2 shows the sucrose step gradient that was finally adopted to produce three microsomal fractions which were enriched in plasma membrane (Band I), Golgi membrane (Band II) and RER-membrane (Band III) markers. The development of these fractions in a fixed angle rotor is relatively unusual, however, centrifugation times were short and there were no problems with re-orientation of tubes provided they were not overloaded. The fractions were characterised using a number of traditional marker enzymes and were assessed for purity against previously characterised membrane fractions produced as shown in Figure 3.1.

An initial low-speed centrifugation at $1,500g_{av}$ removed nuclei and unbroken cells. This 'nuclear' fraction was discarded and all enrichment and purification calculations refer to the 'post-nuclear' supernatant. Twelve fractions (designated in figure 3.1) were isolated and assayed for marker enzyme activities. Previous studies had shown that low speed differential centrifugation removes in addition to chromaffin granules, both mitochondria and lysosomes, and to some extent microsomal (ie. Golgi and RER membrane) components (Banks, 1965). Much of the plasma membrane is also removed by this procedure (Trifaró and Duerr, 1976). Thus, by exploiting differential sedimentation under conditions where organelles should not fragment before exposing microsomal membranes to osmotic shocks or pelleting, the contribution to the microsomal

Figure 3.2. Fractionation of Adrenal Medullary Microsomes By Flotation on a Step Gradient of Sucrose.




Microsomal fractions were separated on a step gradient of sucrose by resuspending membranes in 1.4M-sucrose and loading under gradients of 0.5M/0.86M/1.15M-sucrose poured in polycarbonate tubes. After centrifugation for 60min at 45,000rpm in a Beckman 45Ti rotor the membranes were collected from the interfaces as shown, were washed by dilution to isotonicity and centrifugation. For operational purposes the fractions were termed Bands I, II & III as shown.

fraction by membranes other than those of an endomembrane origin was minimised.

Differential centrifugation at 18,000$g_{av}$ produced a pellet containing 78% of the cytochrome oxidase activity (Table 3.3) and 45% of the content of cytochrome $b_{561}$, markers for mitochondrial inner membrane and chromaffin granule membranes respectively. The latter was measured by difference spectroscopy of crude material, which may lack specificity for this cytochrome, accounting for its apparent presence in soluble fractions. The pellet also contained 30% of the acid precipitable RNA, suggesting that a substantial proportion of the RER may have sedimented. Of the 44% detected in the 'post-secretory granule' supernatant only 10% of the total acid precipitable RNA was recovered in the microsomal pellets.

After separation and recovery from the step gradient of sucrose the microsomal fractions were diluted with buffer, recovered by high speed centrifugation and finally resuspended in buffered 0.3M-sucrose for analysis. They were enriched in many of the markers shown in Table 3.3; in particular there was a 2-fold enrichment in galactosyltransferase. Although a residual 'light membrane' fraction isolated as a by-product of the purification of mitochondrial membranes showed a 6-fold enrichment in galactosyltransferase over that measured in the post-nuclear supernatant, it will become clear below why this fraction was not used to purify Golgi membranes.

Table 3.3. Distribution of Enzyme Marker Activities After Fractionation of Adrenal Medullary Homogenates by Differential and Isopycnic Centrifugation.

| | Fraction | Protein Recovery (%) | Galactosyltransferase Spec.Act. (nmol/h/mg) | Enrichment (fold) | Recovery (%) | Acetylcholinesterase Spec.Act. (nmol/min/mg) | Enrichment (fold) | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| 1. | Post-nuclear Supernatant. | 100 (4) | 4.3±0.9 (4) | 1.0 | 100 | 57.7±13.1 (4) | 1.0 | 100 |
| 2. | Post-Granule Supernatant. | 55.8±17.3 (4) | 2.0±0.6 (4) | 0.5 | 26.0 | 37.2±10.6 (4) | 0.7 | 36.0 |
| 3. | Granule+Mito. Pellet. | 33.3±7.2 (3) | 4.8±1.7 (3) | 1.1 | 37.0 | 52.1±13.6 (3) | 0.9 | 30.1 |
| 4. | Post-Micro. Supernatant. | 38.0±5.7 (3) | 1.3±0.4 (3) | 0.3 | 11.5 | 15.1±4.3 (3) | 0.3 | 10.0 |
| 5. | Microsomal Pellet. | 4.9±1.5 (4) | 8.5±4.0 (4) | 2.1 | 9.7 | 91.1±27.7 (4) | 1.6 | 7.7 |
| 6. | Band I. | 0.06±0.02 (4) | 25.8±3.9 (4) | 6.0 | 0.4 | 380±114 (4) | 6.7 | 0.4 |
| 7. | Band II. | 0.21±0.01 (4) | 27.1±7.1 (4) | 6.3 | 1.3 | 257±79 (4) | 4.5 | 0.9 |
| 8. | Band III. | 0.27±0.1 (4) | 11.1±7.6 (4) | 2.6 | 0.7 | 52.4±25.6 (4) | 0.9 | 0.3 |
| 9. | Granule Membranes. | 1.4±0.8 (4) | 1.0±0.3 (4) | 0.3 | 0.3 | 20.9±11.6 (4) | 0.4 | 0.5 |
| 10. | Mitochondrial Membranes. | 4.5±2.3 (4) | 11.5±6.0 (4) | 2.7 | 12.0 | 45.0±15.5 (4) | 0.8 | 3.5 |
| 11. | Light Membranes. | 1.2±0.3 (4) | 27.1±11.7 (4) | 6.3 | 8.2 | 94.5±57.7 (4) | 1.6 | 2.0 |
| 12. | Granule Lysate. | 6.6±1.6 (4) | 2.0±1.2 (4) | 0.5 | 3.1 | 19.4± 0.3 (1) | 0.3 | 2.2 |

Enzyme marker analysis of twelve subcellular fractions described in figure 3.1. The results are presented as the sample standard deviation ( n-1) and the figures in parenthesis are the number of different subcellular fractionations which were analysed. Washes of the membrane fractions were discarded.

Table 3.3. (cont.) Distribution of Enzyme Marker Activities After Fractionation of Adrenal Medullary Homogenates by Differential and Isopycnic Centrifugation.

| Fraction | Glucose-6-Phosphatase | | | β-Glycerophosphate | | | Ribonucleic Acid | | |
|---|---|---|---|---|---|---|---|---|---|
| | Spec.Act. (nmol/min/mg) | Enrichment (fold) | Recovery (%) | Spec.Act. (nmol/min/mg) | Enrichment (fold) | Recovery (%) | Content (µg/mg) | Enrichment (fold) | Recovery (%) |
| 1. PNS. | 9.3±1.8 (3) | 1.0 | 100 | 7.6±1.9 (3) | 1.0 | 100 | 24±12 (3) | 1.0 | 100 |
| 2. PGS. | 10.6±2.4 (3) | 1.1 | 63.6 | 7.7±2.0 (3) | 1.0 | 56.5 | 19±9 (4) | 0.8 | 44.2 |
| 3. GMP. | 9.7±3.9 (3) | 1.0 | 34.7 | 8.6±5.3 (3) | 1.1 | 37.7 | 22±9 (2) | 0.9 | 30.5 |
| 4. PMS | 10.0±1.3 (3) | 1.1 | 40.9 | 8.9±2.1 (3) | 1.2 | 44.5 | 10±4 (3) | 0.4 | 15.8 |
| 5. MP. | 9.0±3.3 (3) | 1.0 | 4.7 | 4.4±2.0 (3) | 0.6 | 2.8 | 47±9 (4) | 2.0 | 9.6 |
| 6. BI. | 14.5±6.2 (3) | 1.6 | 0.1 | ND (3) | - | - | 23±6 (3) | 1.0 | 0.1 |
| 7. BII. | 28.8±12.7 (3) | 3.1 | 0.7 | 5.0±0.7 (3) | 0.7 | 0.1 | 24±6 (5) | 1.0 | 0.2 |
| 8. BIII. | 10.1±4.9 (3) | 1.1 | 0.3 | 3.0±0.7 (3) | 0.4 | 0.1 | 52±12 (5) | 2.2 | 0.6 |
| 9. CGM. | 2.7±2.0 (3) | 0.3 | 0.4 | 0.8±0.8 (3) | 0.1 | 0.2 | 16±5 (3) | 0.7 | 0.9 |
| 10. MM. | 4.9±4.8 (3) | 0.5 | 2.4 | 1.8±1.7 (3) | 0.2 | 1.1 | 42±12 (3) | 1.8 | 7.9 |
| 11. LM. | 5.3±3.9 (3) | 0.6 | 0.7 | 1.5±1.8 (3) | 0.2 | 0.2 | 23±6 (3) | 1.0 | 1.2 |
| 12. CGL. | 6.3±5.5 (3) | 0.7 | 4.5 | 4.8±5.0 (3) | 0.6 | 4.2 | - | - | - |

Table 3.3. (cont.) Distribution of Enzyme Marker Activities After Fractionation of Adrenal Medullary Homogenates by Differential and Isopycnic Centrifugation.

| Fraction | 5'-Nucleotidase | | | 2',3'-Nucleotidase | | | Acid Phosphatase. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Spec.Act. (nmol/min/mg) | Enrichment (fold) | Recovery (%) | Spec.Act. (nmol/min/mg) | Enrichment (fold) | Recovery (%) | Spec.Act. (nmol/min/mg) | Enrichment (fold) | Recovery (%) |
| 1. PNS. | 7.8±4.1 (3) | 1.0 | 100 | 3.7 (1) | 1.0 | 100 | 10.6 (1) | 1.0 | 100 |
| 2. PGS. | 8.0±3.3 (3) | 1.0 | 57.2 | 4.5 (1) | 1.2 | 67.9 | 34.3 (1) | 3.2 | 303 |
| 3. GMP. | 9.5±6.2 (3) | 1.2 | 40.6 | 5.3 (1) | 1.4 | 47.7 | 6.7 (1) | 0.6 | 23.7 |
| 4. PMS | 8.3±3.1 (3) | 1.1 | 40.4 | 8.7 (1) | 2.4 | 89.4 | 46.5 (1) | 4.4 | 411 |
| 5. MP. | 4.9±3.1 (3) | 0.6 | 3.1 | 2.0 (1) | 0.5 | 2.7 | 9.6 (1) | 0.9 | 21.5 |
| 6. BI. | 12.7±2.8 (3) | 1.6 | 0.1 | 2.8 (1) | 0.8 | 0.1 | 31.7 (1) | 3.0 | 1.4 |
| 7. BII. | 10.1±5.7 (3) | 1.3 | 0.3 | 3.5 (1) | 1.0 | 0.2 | 11.9 (1) | 1.1 | 0.7 |
| 8. BIII. | 4.1±2.0 (3) | 0.5 | 0.1 | 1.4 (1) | 0.4 | 0.1 | 34.4 (1) | 3.3 | 2.0 |
| 9. CGM. | 0.8±0.3 (3) | 0.1 | 0.1 | 0.4 (1) | 0.1 | 0.2 | ND (1) | - | - |
| 10. MM. | 5.6±4.9 (3) | 0.7 | 3.2 | 2.3 (1) | 0.6 | 2.8 | 18.2 (1) | 1.7 | 1.0 |
| 11. LM. | 4.9±2.3 (3) | 0.6 | 0.8 | 1.1 (1) | 0.3 | 0.4 | 11.0 (1) | 1.1 | 1.9 |
| 12. CGL. | 2.5±2.2 (3) | 0.3 | 2.1 | 6.0 (1) | 1.6 | 10.7 | 15.3 (1) | 1.4 | 95 |

Table 3.3. (cont.) Distribution of Enzyme Marker Activities After Fractionation of Adrenal Medullary Homogenates by Differential and Isopycnic Centrifugation.

| Fraction | Cytochrome Oxidase. Spec.Act. (nmol/min/mg) | Enrichment (fold) | Recovery (%) | Cytochrome P450. Spec.Act. (pmol/mg) | Enrichment (fold) | Recovery (%) | Cytochrome b561. Spec.Act. (nmol/mg) | Enrichment (fold) | Recovery (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1. PNS. | 89.6±17.5 (3) | 1.0 | 100 | ND (1) | - | - | 0.5 (1) | 1.0 | 100 |
| 2. PGS. | 3.6±0.7 (3) | 0.1 | 2.2 | ND (1) | - | - | 0.3 (1) | 0.5 | 24 |
| 3. GMP. | 209±6 (3) | 2.3 | 77.7 | 113 (1) | 1.0 | 100 | 0.6 (1) | 1.2 | 45 |
| 4. PMS | 2.3±1.1 (3) | 0.1 | 1.0 | ND (1) | - | - | 0.4 (1) | 0.7 | 29 |
| 5. MP. | 19.4±6.5 (3) | 0.2 | 0.2 | 60 (1) | 0.5 | 16.1 | ND (1) | - | - |
| 6. BI. | 7.1±3.7 (3) | 0.1 | 0.1 | ND (1) | - | - | 0.4 (1) | 0.8 | 0.1 |
| 7. BII. | 34.7±14.6 (3) | 0.4 | 0.1 | 55 (1) | 0.5 | 0.2 | 0.4 (1) | 0.8 | 0.1 |
| 8. BIII. | 56.4±27.8 (3) | 0.6 | 0.2 | 27 (1) | 0.2 | 0.2 | ND (1) | - | - |
| 9. CGM. | 75.4±19.3 (3) | 0.8 | 1.2 | 46 (1) | 0.4 | 1.1 | 3.0 (1) | 5.7 | 14.5 |
| 10. MM. | 761±429 (3) | 8.5 | 38.2 | 203 (1) | 1.8 | 30 | ND (1) | - | - |
| 11. LM. | 60.1±23.7 (3) | 0.7 | 0.8 | 49 (1) | 0.4 | 1.4 | 0.4 (1) | 0.8 | 0.7 |
| 12. CGL. | ND (3) | - | - | ND (1) | - | - | ND (1) | - | - |

## 1. Galactosyltransferase: Golgi Membranes.

UDP-galactose 4B-galactosyltransferase, (EC 2.4.1.38) is membrane bound in tissues and uniquely located in the trans-cisternae of the Golgi complex. Soluble forms of the enzyme are present in milk (Babad and Hassid, 1966) and serum (Wagner and Cynkin, 1971). Use of galactosyltransferase as a marker for the Golgi complex assumes retention of this organelle's stacked membrane morphology during subcellular fractionation; in the absence of confirmation of such morphology by electron microscopy this enzyme is therefore only a marker for the trans-most cisternal membranes of the Golgi complex.

Golgi-enriched microsomal fractions have been isolated from adrenal medullary tissue, using galactosyltransferase as a marker (Trifaró and Duerr, 1976). However, there is no published characterisation of the enzyme from this tissue. An assay was therefore developed and optimised with both microsomal and Golgi-enriched microsomal fractions.

Under the conditions given in Chapter 2 (Page 71) the incorporation of [$^3$H]-galactose into ovomucoid was linear for at least 60min (Figure 3.3a) and was proportional to the amount of protein in Golgi-enriched membranes (Figure 3.3b). Galactosyltransferase requires $Mn^{2+}$ for maximal activity (Schachter et al., 1970; McGuire et al., 1965); this was achieved at concentrations above 15mM for the adrenal medullary enzyme (Figure 3.3c). Some galactosyltransferases express activity with $Mg^{2+}$ as cofactor, although this was not required for the adrenal medullary enzyme. Glycoprotein acceptor saturation was at 5mg/ml of ovomucoid (Figure 3.3d). The contribution from endogenous acceptors in Golgi-enriched fractions was assayed with the concentration of

FIGURE 3.3. Galactosyltransferase Activity.

(a). **Time Course of the Transfer of [$^3$H]-Galactose to Ovomucoid by Golgi-Enriched Membranes.**

The assay medium (as described on page 73) contained ovomucoid at 5mg/ml and enzyme at 0.25mg of protein/ml. At the times indicated 100µl samples were withdrawn and added to 2ml of ice-cold 10% TCA and processed for scintillation counting. There was no significant incorporation of radiolabel into endogenous acceptors (see text). The specific activity of galactosyltransferase in this experiment was 38nmol/hr/mg of protein.

(b). **Transfer of [$^3$H]-Galactose as a Function of Golgi Membrane Protein.**

Increasing amounts of Golgi enriched membranes (Fraction 7) were added from stocks at 0.2-2mg of protein/ml as shown and incubated for 60min at 37$^o$C . The reaction was stopped with 10%TCA and the sample processed for scintillation counting.

(c). **Galactosyltransferase Activity as a Function of the Manganese Concentration.**

The figure shows the requirement of manganese ions for the expression of galactosyltransferase activity in Golgi-enriched membranes (0.26mg of protein/ml); incubation and processing of samples was as described above.

(d). **Galactosyltransferase Activity as a Function of Glycoprotein Acceptor (Ovomucoid) Concentration.**

Golgi-enriched membranes (0.26mg of protein/ml) were assayed for galactosyltransferase activity in the presence of various concentrations of ovomucoid (added from a 100mg/ml stock solution) as shown. The incubation was at 37$^o$C for 60min, the reaction was stopped with TCA and samples processed for scintillation counting.

(e). **Activation of Galactosyltransferase by Dithiothreitol.**

Galactosyltransferase activity of Golgi-enriched membranes (o.26mg of protein/ml) were assayed in the presence of increasing concentrations of DTT as shown.

(f). **pH-Dependence of Golgi Enriched Membrane Galactosyltransferase.**

Golgi-enriched membranes (0.26mg of protein/ml) were incubated at 37$^o$C at the given pH for 60min in 50mM of the following buffers: **•**, Mes (pH5.23-6.49); **o**,Mops (pH6.19-7.49); **x**,Hepes (pH6.43-7.85). The pH of diluted stock buffers at 50mM was measured at 37$^o$C.

(g). **The Effect of Sucrose on the Galactosyltransferase Activity of Golgi Membranes.**

Golgi-enriched membranes (0.16mg of protein/ml) were assayed in the presence of increasing concentrations of sucrose which reflected those present in fractions from sucrose gradients.

Figure 3.3a

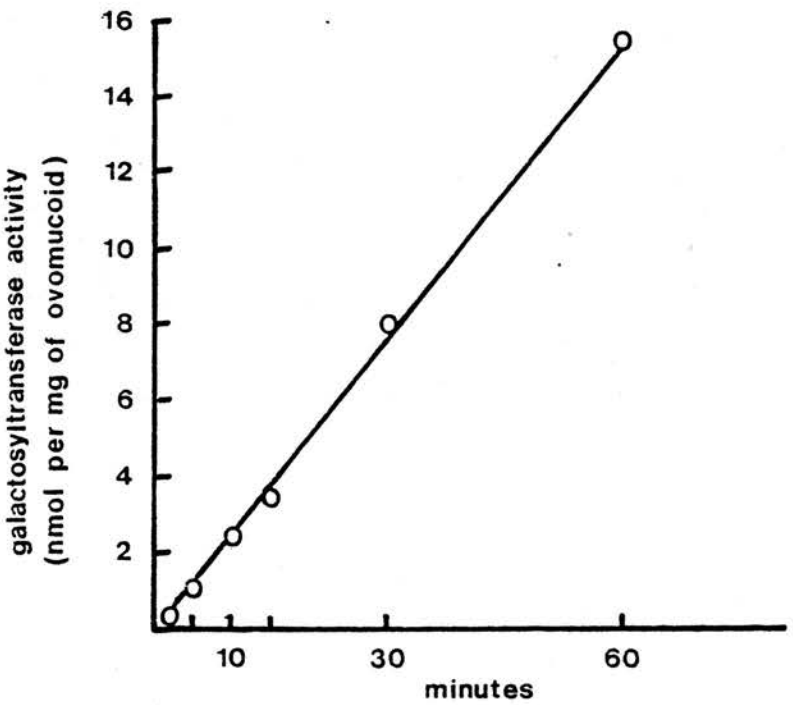



Figure 3.3b

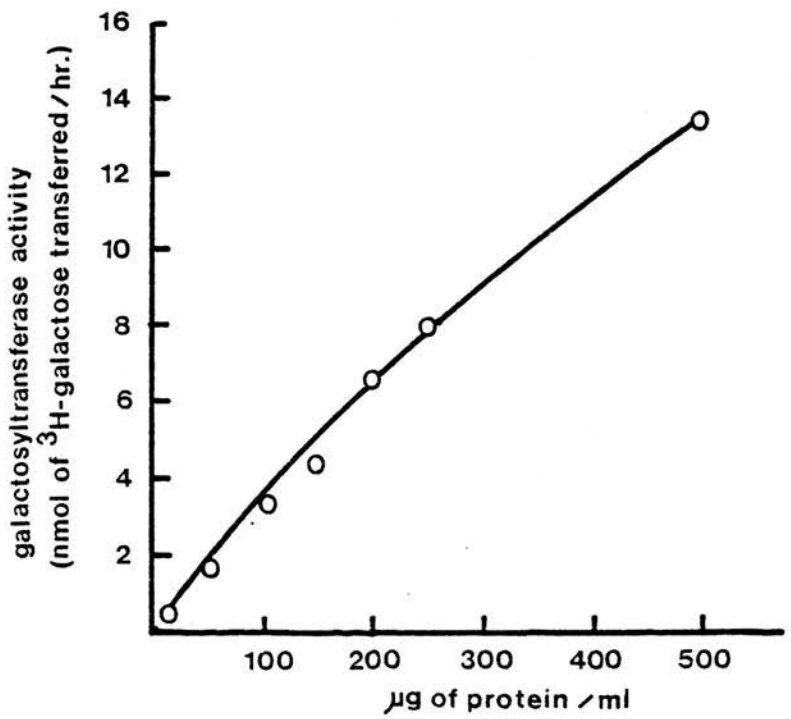


Figure 3.3c

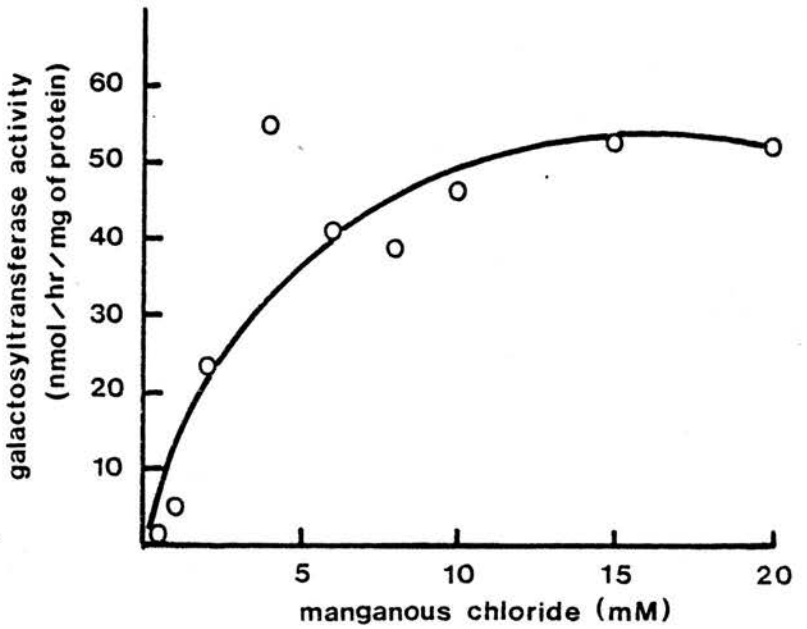



Figure 3.3d

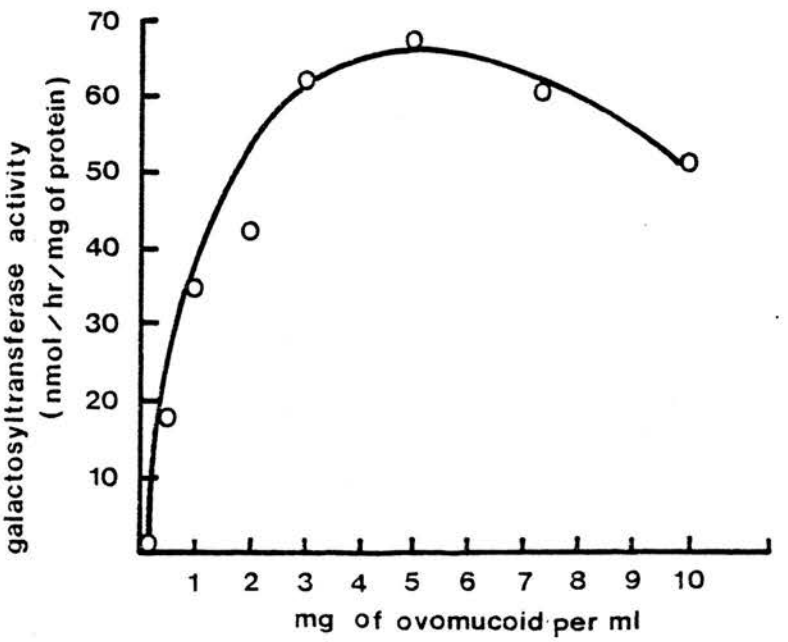


Figure 3.3e

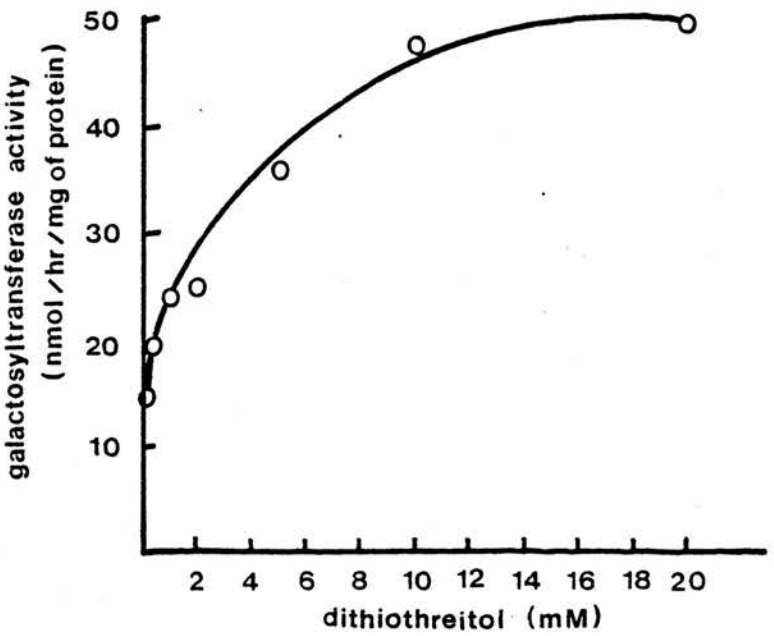



Figure 3.3f

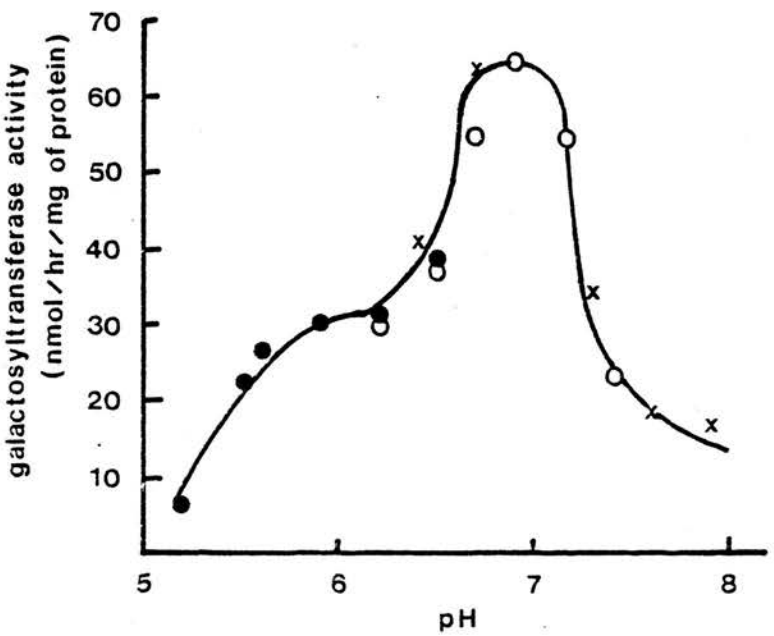
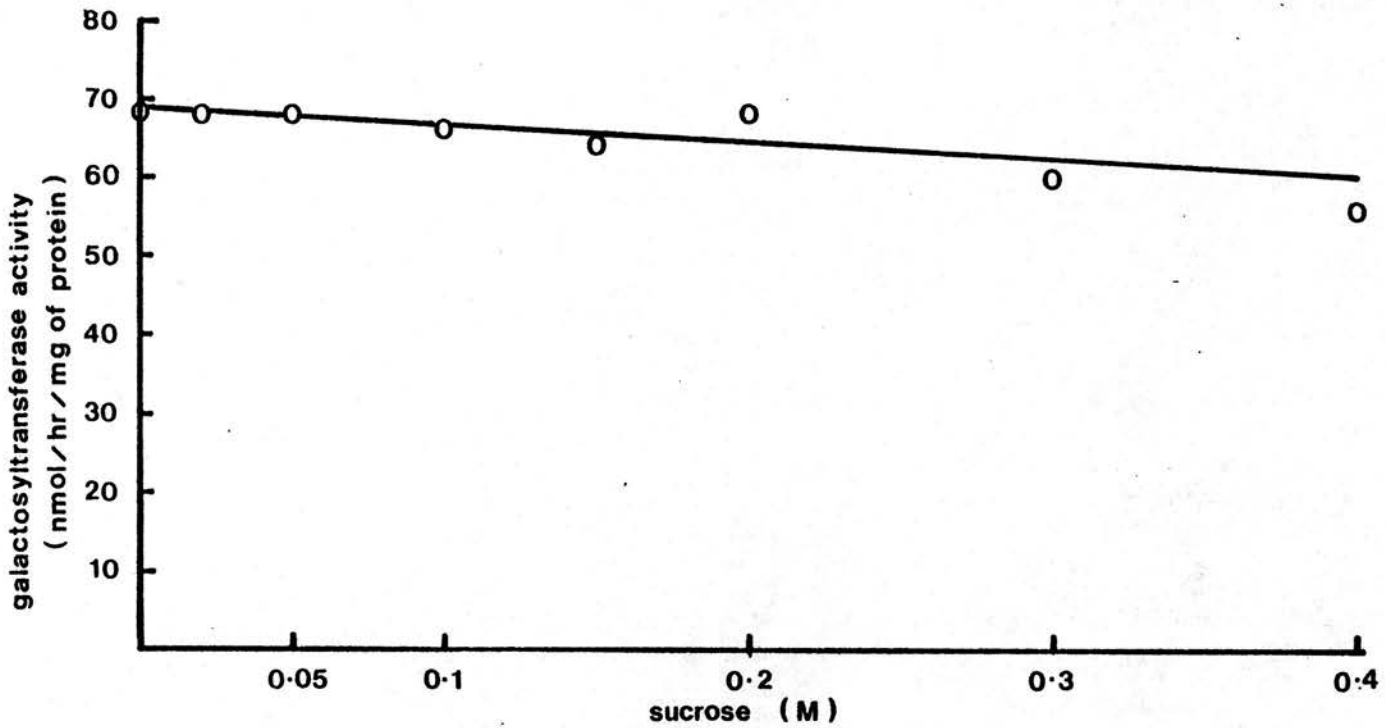


Figure 3.3g

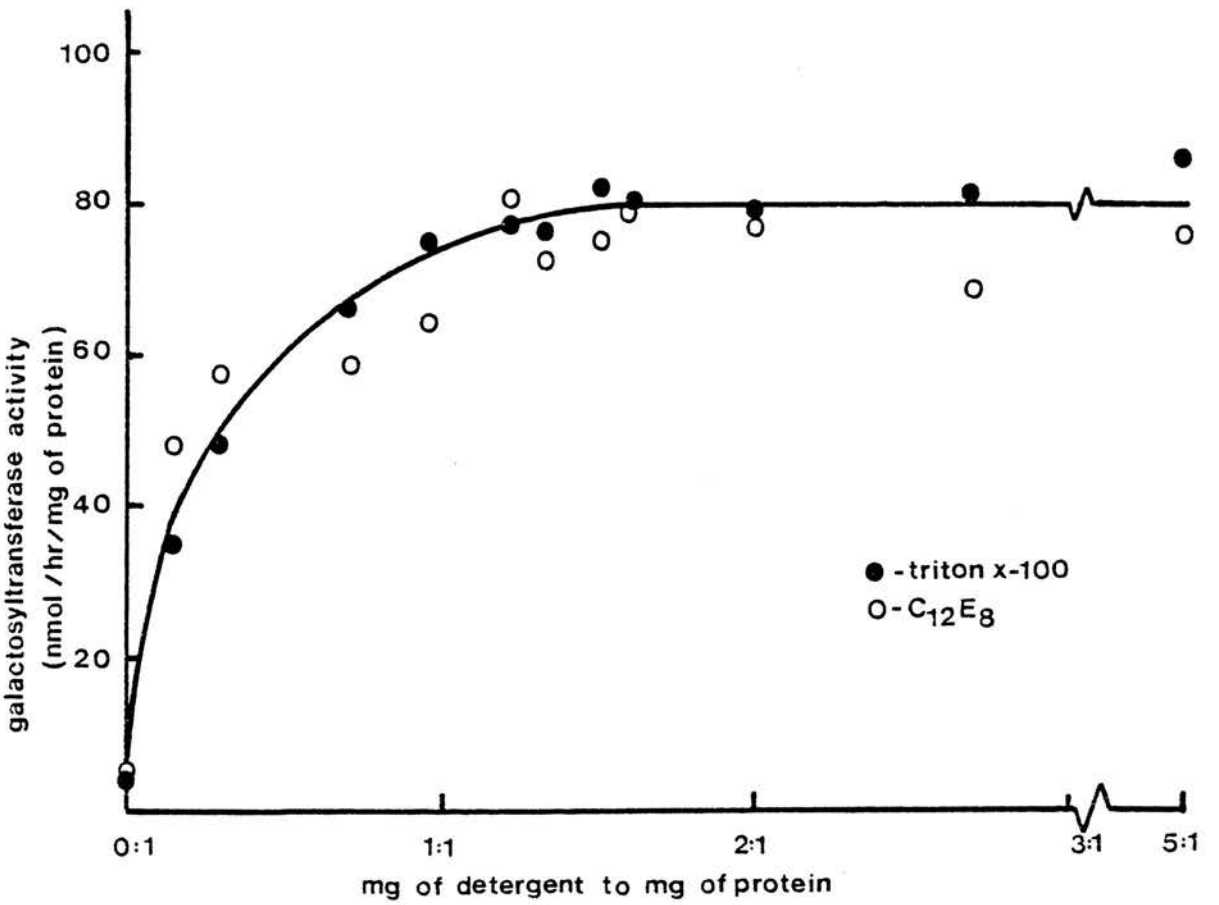

80
70
60
50
40
30
20
10
galactosyltransferase activity
( nmol / hr / mg of protein )
0·05
0·1
0·2
0·3
0·4
sucrose (M)

the [$^3$H]UDP-galactose raised to 10μCi/ml. Its incorporation, monitored over 4hrs, was negligible under these conditions; the specific activity was 0.29nmol/hr/mg of protein, while in the presence of ovomucoid this was raised 234-fold to 68nmol/hr/mg of protein. Like the rat liver enzyme (Schachter et al., 1970) adrenal medullary galactosyltransferase activity is stimulated (3-fold) in the presence of DTT (Figure 3.3e).

Figure 3.3f shows the pH-profile for galactosyltransferase in Golgi-enriched membranes. It is optimal between pH6.8 and 7. In addition there was a minor activity at pH6, the nature of which is unknown. However, galactosyltransferases with pH optima at both 6 and 7 have been identified in rat liver fractions (Kaplan and Hechtman, 1983) and a 1,3-galactosyltransferase which has a pH optimum at 6 may be involved in addition of galactose to O-linked carbohydrate chains (Sheares and Carlson, 1983). This enzyme, having a pH optimum at 6, may be responsible for the addition of galactose to the chromogranins. Because many subcellular fractions were assayed directly from sucrose gradients the effect of sucrose (and its contaminants) on galactosyltransferase activity was assessed (Figure 3.3g). Sucrose solutions were only inhibitory at high concentrations; there was always less than 0.2M-sucrose in assay mixes. When stored at -20$^o$C adrenal medullary galactosyltransferase activity was stable.

The active site of galactosyltransferase is on the luminal side of the Golgi membrane (Fleischer, 1981; Strous et al, 1983b). Figure 3.4 shows that in the absence of detergent there is no significant transfer of [$^3$H]-galactose to ovomucoid. This is a powerful indication that the structural integrity of Golgi vesicles has been maintained during subcellular fractionation; 96% of the

FIGURE 3.4. Latency of Galactosyltransferase Activity.




Golgi-enriched membranes (Fraction 7) (0.74mg of protein/ml) were solubilised with varying amounts of Triton X-100 and $C_{12}E_8$ at 0°C and samples were taken to assay for galactosyltransferase activity (37°C; 60min). Final protein concentration in the assay was 0.15mg/ml. Results are presented as the detergent to protein ratio (mg/mg) vs enzyme specific activity.

enzyme activity remained latent. For routine monitoring during subcellular fractionation membranes were solubilised by including 0.5% (w/v) $C_{12}E_8$ in the assay medium.

Previous studies with adrenal medullary fractions enriched in galactosyltransferase (Duerr et al., 1974; Trifaró and Duerr, 1976; Trifaró et al., 1976), have used N-acetylglucosamine as the acceptor molecule for galactose rather than a glycoprotein acceptor and have reported Golgi-membrane fractions enriched 8- to 14-fold with specific galactosyltransferase activities of up to 150nmol/hr/mg of protein. In this study, with ovomucoid as acceptor, specific activities of between 25-80nmol/hr/mg of protein, with a 6- to 8-fold enrichment over a 'post-nuclear' supernatant fraction were obtained. Thirtyseven percent of the galactosyltransferase activity was recovered in the crude chromaffin granule pellet (Table 3.3) and of this the majority remained associated with fractions derived from crude mitochondrial membranes (see Figure 3.1). Thus it appears that some stacks of Golgi membrane may be lost during low speed centrifugation; a consequence of the gentle homogenisation procedure used. Fraction 11 of Table 3.3 (see also Figure 3.1) showed an enrichment (6-fold) in galactosyltransferase activity equivalent to that of the 'Golgi-enriched' microsomal fraction (Fraction 7). However, it was contaminated with relatively large amounts of plasma membrane and contained ten times more cytochrome oxidase activity than the Golgi-enriched microsomal fraction; these contaminating membranes would have co-sedimented with the Golgi membranes on further isopycnic centrifugation. It was this type of fraction, exposed to osmotic shock and pelleting that was used by Duerr and Trifaró (1976). Such a fraction was not suitable for the type of electrophoretic analysis to be undertaken here (Chapter 4), and

indeed, two-dimensional gel analysis of Fraction 11 gave a polypeptide pattern dominated by chromaffin granule and mitochondrial proteins (not shown). The post microsomal supernatant contained over 11% of galactosyltransferase activity without showing enrichment (Table 3.3), almost 10% of this activity was recovered with the microsomal membranes. This fraction was further fractionated on step gradients of sucrose; after recovery of particulate material by centrifugation of the main bands, much of this enzyme activity had been lost from fractions 6, 7 and 8 (Table 3.3). However Band II (fraction 7) contained over 1% of the original galactosyltransferse activity of the post nuclear supernatant and was enriched over 6-fold. Band I was also enriched 6-fold in galactosyltransferase but contained less of the total activity.

2. Glucose-6-phosphatase: RER Membranes.

This enzyme is a marker for rat liver endoplasmic reticulum (Carey and Hirschberg, 1980) and has been used as a marker in the adrenal medulla (Hörtnagl, 1976). However, it has proved to be a poor marker in tissues other than the liver. Post-microsomal adrenal medullary supernatants contained 41% of the apparent glucose-6-phosphatase activity (Table 3.3), only about 5% being associated with the microsomal membranes (Fraction 5). The specific activities reported here for glucose-6-phosphatase (Table 3.3) are similar to previously published estimates (Wilson and Kirshner, 1976). However, when non-specific phosphatase activity was monitored in parallel control assays containing $\beta$-glycerophosphate as substrate, Table 3.3 shows that between 70% and 90% of the activity of glucose-6-phosphatase associated with crude fractions

can be accounted for by non-specific phosphatases.

Assay of acid phosphatase showed only 3% associated with the microsomal fraction. It is a traditional marker for lysosomes, but its enrichment in Band I (Fraction 6; Table 3.3) may indicate an association with the plasma membrane (as in the astrocytomas, Leis and Kaplan, 1982).

In addition to the problem of specificity in assays, both glucose-6-phosphatase and 5'-nucleotidase activities (see below) are progressively lost from membranes during fractionation, suggesting that they may be adherent soluble proteins that redistribute during homogenisation; such contamination problems have been appreciated during subcellular fractionation of rat liver. The only microsomal fraction to show an enrichment in glucose-6-phosphatase was Band II (Fraction 7), the fraction that is also enriched in galactosyltransferase.

Thus it appears that glucose-6-phosphatase is not a good marker for RER in chromaffin tissue. Previous studies have reported no significant enrichment in adrenal medullary microsomal fractions (Wilson and Kirshner, 1976) and while Band III (Fraction 8) showed a 2.2-fold enrichment in acid-precipitable RNA it showed no concomitant enrichment in glucose-6-phosphatase. This enzyme has been associated with Golgi membranes in other tissues and it may be associated with Golgi membrane here. However, the large variation (greater than 44%) about the mean glucose-6-phosphatase activity again suggests contamination by soluble phosphatases.

3. 5'-Nucleotidase and Acetylcholinesterase: Plasma Membrane Markers.

5'-Nucleotidase, traditionally a plasma membrane marker, suffers from the same drawbacks as glucose-6-phosphatase. Hydrolysis of 2,3'-AMP was used as a control for non-specific phosphatase. 5'-nucleotidase shows a slight enrichment in Microsomal Band I (Fraction 6), together with a standard deviation of only 22% of the mean activity (compared with around 50% for other fractions). In spite of this the 'signal-to-noise' ratio is still low, making this an unreliable marker for plasma membrane in this tissue; its value as a marker has also been questioned for other tissues (Oseroff et al., 1973). However, microsomal Band I does show enrichment in both 5'-nucleotidase and acetylcholinesterase, these enzymes having identical distribution coefficients (ie. ratio of acetylcholinesterase to 5'-nucleotidase is 1.5 when the contribution from non-specific activity towards 2,3'-AMP is subtracted) between Band I and Band II (Table 3.3). This is in contrast to the study of Trifaró and Duerr (1976) whose Golgi membranes were enriched in 5'-nucleotidase, and Wilson and Kirshner (1976) who like Zinder et al. (1978) used this enzyme as a plasma membrane marker, but found that 5'-nucleotidase activity did not co-sediment with acetylcholinesterase. The specific activity of 5'-nucleotidase in adrenal medullary tissue in comparison with other tissues is low (Nijjar and Hawthorne, 1974). In some tissues its activity has been associated with RER fractions (Widnell, 1972; Farquhar and Palade, 1981) throwing into doubt its validity as a plasma membrane marker enzyme. Histochemical studies have localised acetylcholinesterase to the plasma membrane of adrenal medullary cells, however some staining may be associated with RER membranes

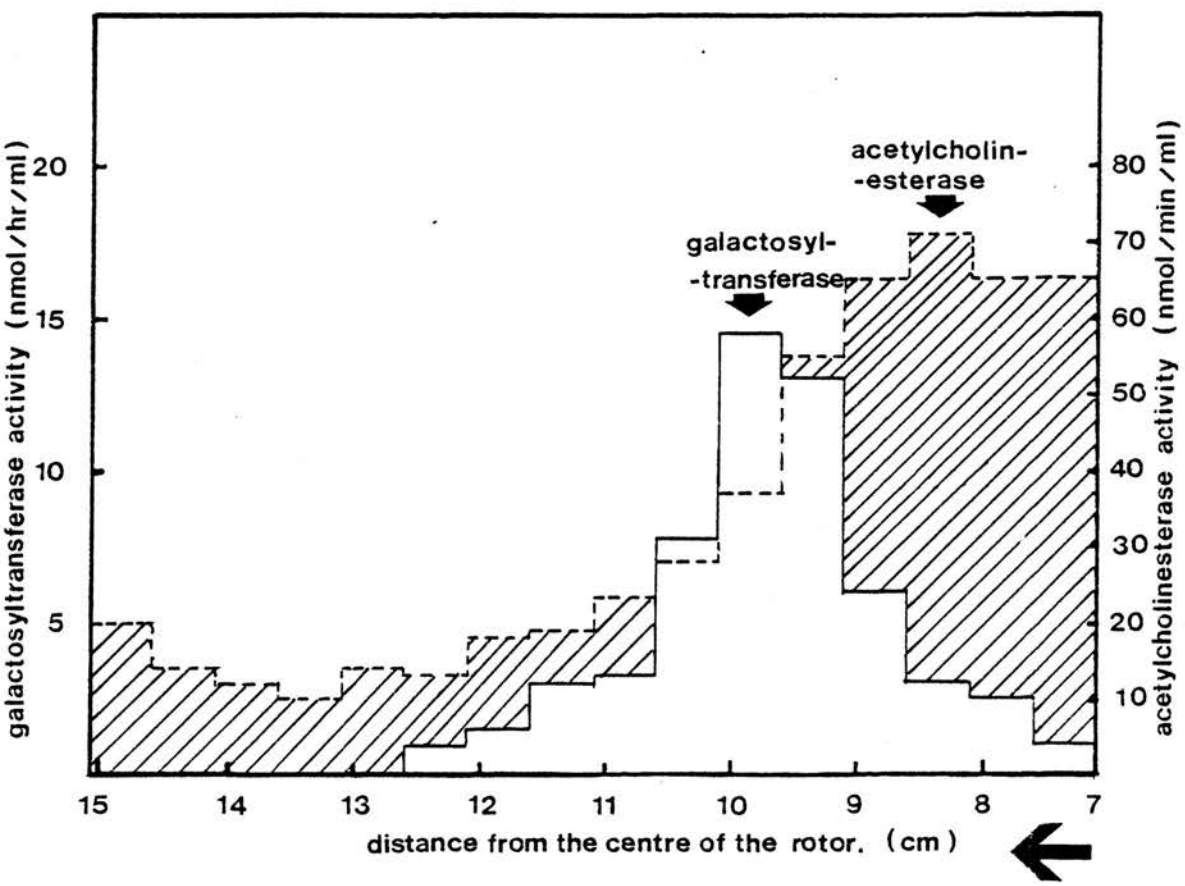
(Nijjar and Hawthorne, 1974). These anomalies may reflect the fractionation of different domains of the plasma membrane, a point that will be discussed in Chapter 4.

Only a small percentage of the plasma membrane is recovered in the microsomal fraction. Much probably sediments with the nuclear fraction. Much of the remaining acetylcholinesterase activity appears to be soluble. Little of the activity associated with the early insoluble fractions remains on further purification. Wilson and Kirshner (1976) purified plasma membrane with a specific acetylcholinesterase activity of 261nmol/min/mg of protein; microsomal Band I (Fraction 6) has a specific activity of 380nmol/min/mg and is therefore designated as a putative purified plasma membrane fraction, but it is clear from Table 3.3 that much must also be present in Band II.

Chromaffin granule fractions show no enrichment in acetylcholinesterase and only 0.5% of the total activity is associated with chromaffin granule membranes. Homogenisation fragments the plasma membrane into a very heterogeneous population of membrane vesicles. The smaller vesicles contribute to the membrane content of the microsomal fraction. The heterogeneity in the peak of acetylcholinesterase on sucrose gradients of adrenal medullary microsomes confirms this (Figures 3.5 & 3.9). Do these membrane vesicles entrap cytoplasmic proteins during homogenisation?

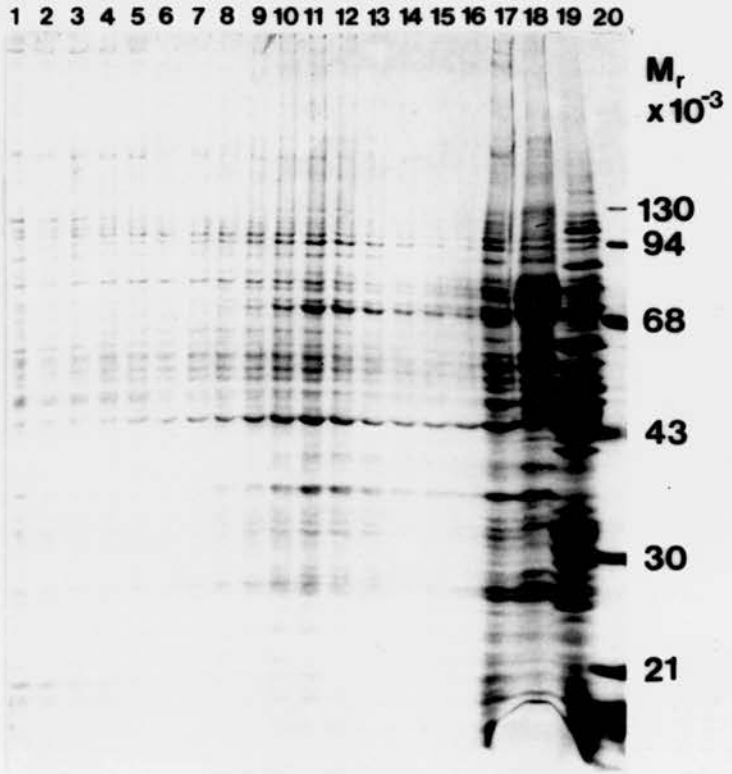
Golgi vesicles are relatively difficult to rupture due to their large membrane to luminal ratio (Howell and Palade, 1982). On the other hand plasma membrane vesicles are very fragile and should release any soluble contents if subjected to hyposmotic conditions: the endomembranes will survive short exposures to hyposmotic conditions with only a small degree of swelling (eg. Green et al.,

FIGURE 3.5. Distribution of Golgi and Plasma Membrane Markers on a Sucrose Gradient Following Hyposmotic Shock of Microsomes.




Analysis of the distribution of microsomal membranes (Fraction 5, Figure 3.1) following their exposure to a hyposmotic shock in 10mM-Hepes NaOH, pH7.2 at 0°C and then rapidly returning the medium to isotonicity with 2M-sucrose. 0.5ml of the suspension (1.55mg) was layered under a 0.5M-1.6M-sucrose gradient. After centrifugation for 15hr at 196,000$g_{av}$ in a Beckman rotor (type SW41Ti) the gradient was fractionated (16x0.75ml) and fractions were assayed for protein, galactosyltransferase and acetylcholinesterase.

FIGURE 3.6. Electrophoretic Analysis of Golgi Membranes After Hyposmotic Shock.




A 6-15% polyacrylamide gel of gradient fractions stained with Coomassie blue. 3vol. of gradient fractions were mixed with 1vol. of [x4] sample buffer containing 8% mercaptoethanol and then 25µl samples were loaded onto the gel. Tracks 1-16 are gradient fractions; track 17, microsomes 50µg; track 18, chromaffin granule membranes 50µg; track 19, mitochondrial membranes 50µg; track 20, standard proteins: β-galactosidase ($M_r$ 130,000), phosphorylase b ($M_r$ 94,000), bovine serum albumin ($M_r$ 68,000), ovalbumin ($M_r$ 43,000), carbonic anhydrase ($M_r$ 30,000), trypsin inhibitor ($M_r$ 21,000), myoglobin ($M_r$ 17,000), lysozyme ($M_r$ 14,300).

1981). To test this, microsomes were subjected to a rapid lytic condition by dilution into 10mM-Hepes NaOH, pH7.4, and then the medium was immediately returned to isotonicity with sucrose. The membranes were fractionated by flotation through a density gradient of sucrose. Figure 3.5 shows that the peak of acetylcholinetrerase activity had now shifted to a lower density (compare with Figure 3.9), apparently giving a better separation of plasma membrane from Golgi membrane. Electrophoretic analysis of these fractions shows that the Golgi membranes (Fractions 11&12; Figure 3.6) have retained their soluble protein content, in particular a polypeptide $M_r$ 68,000 protein which in the next chapter will be shown to characteristic for Golgi membranes. This observation provides indirect evidence that microsomal bands I and II (Figure 3.1; Fractions 6 and 7) are indeed selectively enriched in plasma membrane and Golgi membranes respectively.

4. Glycosidases: RER Markers?

The compartmentation of enzymes which process N-linked glycoproteins make them potentially powerful markers. The glycosyltransferases meet many of the critera for good marker enzymes; many of them, however, require complex substrates and protocols for assay. Processing of glycoproteins also involves glycosidases that appear to be unique to the pathway involved in glycoprotein modification and these enzymes can be biochemically distinguished from their lysosomal counterparts involved in glycoprotein catabolism. The activities can be partially differentiated by their pH optima, and so $\alpha$-glucosidase and $\alpha$-mannosidase were explored as possible markers.

a. Neutral Glucosidase.

Two $\alpha$-glucosidase activities which trim N-linked oligosaccharides are located on the luminal surface of the RER (Waechter and Lennarz, 1976). To ascertain whether $\alpha$-glucosidase activity towards p-nitrophenyl glucoside would be a suitable marker for RER membranes this activity was characterised for adrenal medullary microsomes.

Microsomal $\alpha$-glucosidase activity was proportional to both the time of incubation (Figure 3.7a) and to the amount of protein (Figure 3.7b). Sucrose (Figure 3.7c) and nonionic detergents were shown to be inhibitory (Figure 3.7a). There was no activity towards pnp-$\beta$-D-glucoside (Figure 3.7a). Fractions were washed with 50mM-Hepes NaOH, pH7 to remove sucrose from gradient fractions. The adrenal medullary microsomal activity towards pnp-$\alpha$-D-glucoside had a broad pH optimum around 7 (Figure 3.7d). There was no apparent contribution from acidic glucosidase activity attributable to lysosomal enzymes. Microsomal fractions had an $\alpha$-glucosidase specific activity of 97.2 ±38.6 nmol/hr/mg of protein (n=5). From Hanes plots of [S]/V vs [S] a dissociation constant (Km) of 1.1mM and Vmax of 0.105nmol/hr/$\mu$g of protein were estimated. The analysis suggested that there were possibly more components to the plots. When the RER-enriched fraction (fraction 8) was analysed a high Km (1.75mM) form of the enzyme with a higher Vmax (0.264nmol/hr/$\mu$g) and a less active species (Vmax 0.052nmol/hr/$\mu$g) with a lower Km (0.2mM) were detected.

Glucosidase I has little affinity for pnp-$\alpha$-D-glucoside (Hettkamp et al., 1984) and lysosomal acid $\alpha$-glucosidase has a pH optimum at 4.5 (Jeffery et al., 1970), therefore the adrenal medullary activity appears to be restricted to the membranes of the

Figure 3.7. Adrenal Medullary -Glucosidase.

(a). Time Course of the Hydrolysis of p-Nitrophenol α-D-Glucoside by Adrenal Medullary Microsomes.

Microsomal membranes (Fraction 5, Figure, 3.1) were diluted into 25vol. of 50mM-Hepes NaOH, pH7 and then recovered by centrifugation at 227,000g$_{av}$ for 60min in a Beckman rotor (type 50.2Ti) and suspension in 10mM-Hepes NaOH, pH7. Washed membranes at 1.8mg of protein/ml were incubated for 60min at 37°C and assayed for glucosidase activity as described in Chapter 2. After quenching with 0.2M-$Na_2CO_3$ the release of p-nitrophenol from pnp-α-D-glucoside was estimated spectrophotometrically.

(i). o—o - pnpα-D-glucoside as substrate.

(ii). ●—● - pnpβ-D-glucoside as substrate.

(iii). x—x - Triton X-100.

(iv). ■—■ - $C_{12}E_8$.

(b). α-Glucosidase Activity as a Function of Protein.

Microsomal membranes (Fraction 5, Figure 3.1) washed in 10mM-Hepes NaOH, pH7 were assayed at 37°C for 60min. as described above. Control samples were run in parallel with the enzyme added after $Na_2CO_3$.

(c). Inhibition of α-Glucosidase Activity by Sucrose.

Washed microsomal membranes (0.53mg of protein/ml) were assayed in the presence of increasing concentrations of sucrose under the conditions described above in (a).

(d). pH-Profile of Microsomal Membrane α-Glucosidase activity.

Microsomal membranes (0.34mg of protein/ml) were incubated at 37°C at the given pH for 60min in 50mM of the following buffers: citrate (pH3-5.4); Mes (pH5.5-6.8); Mops (pH6.5-7.8); Hepes (pH6.8-8.2). The reaction was quenched with $Na_2CO_3$ and the release of p-nitrophenol estimated spectrophotometrically. A parallel control incubation with substrate absentwas quenched with $Na_2CO_3$ containing pnp-glycoside. The pH of the [x2]-buffers (100mM) was measured at 37°C.

Figure 3.7a

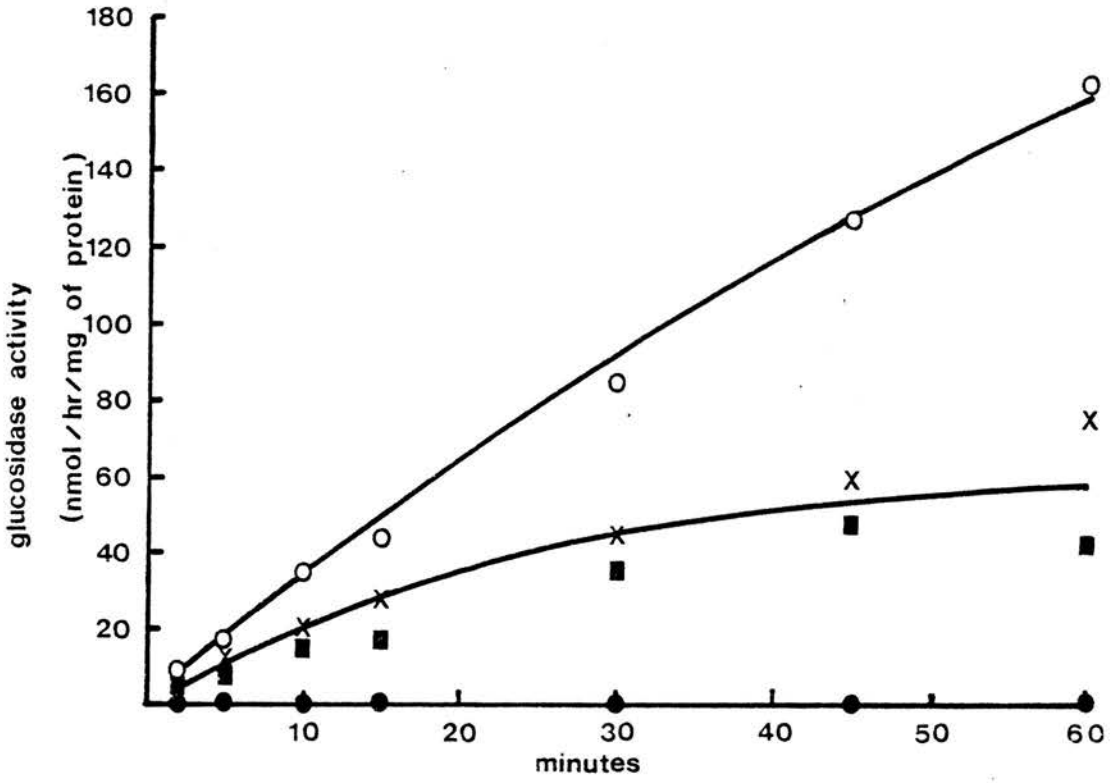



Figure 3.7b

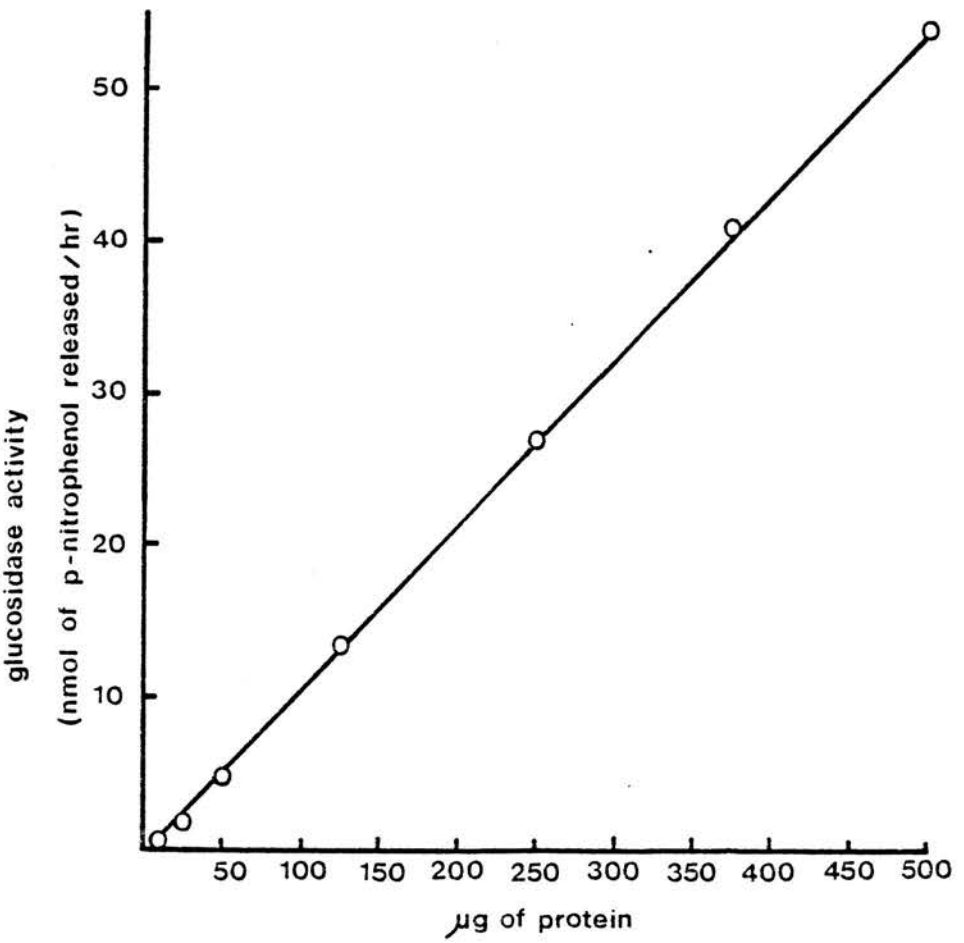


Figure 3.7c

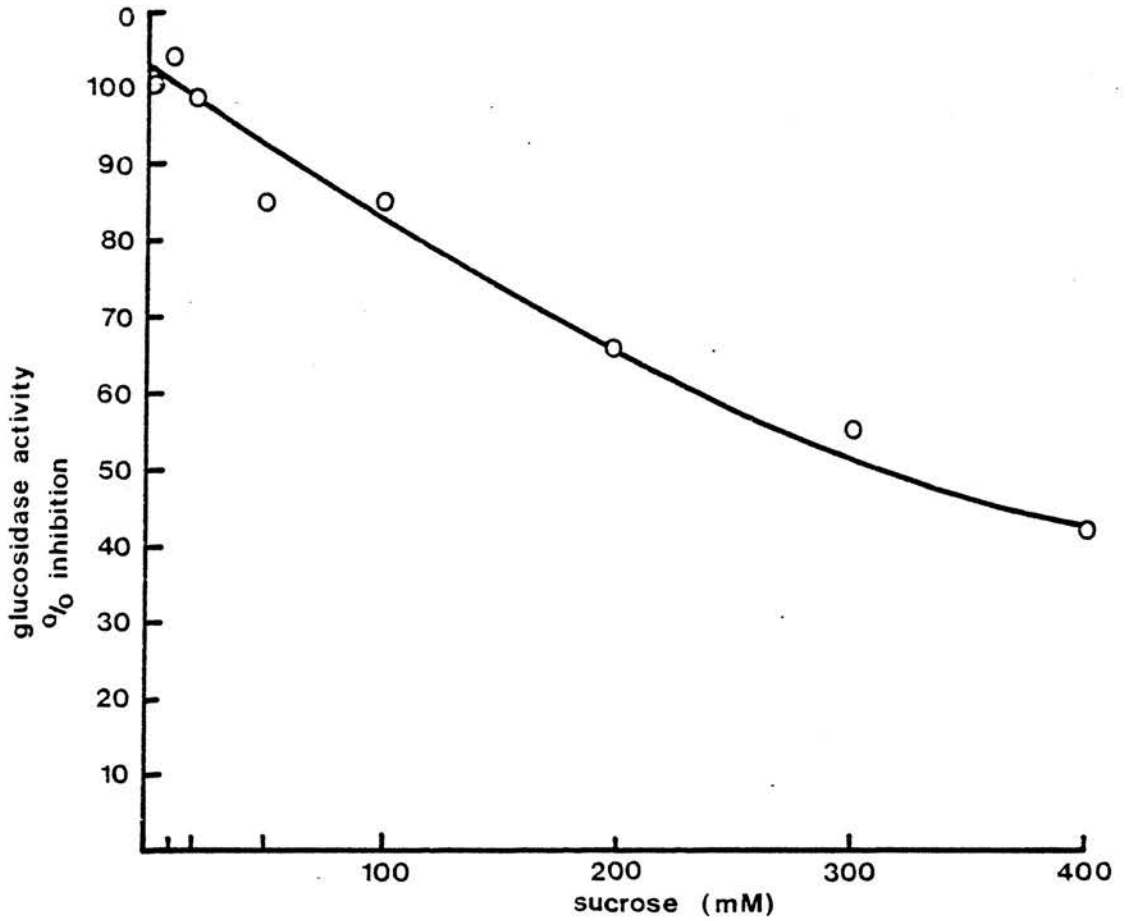
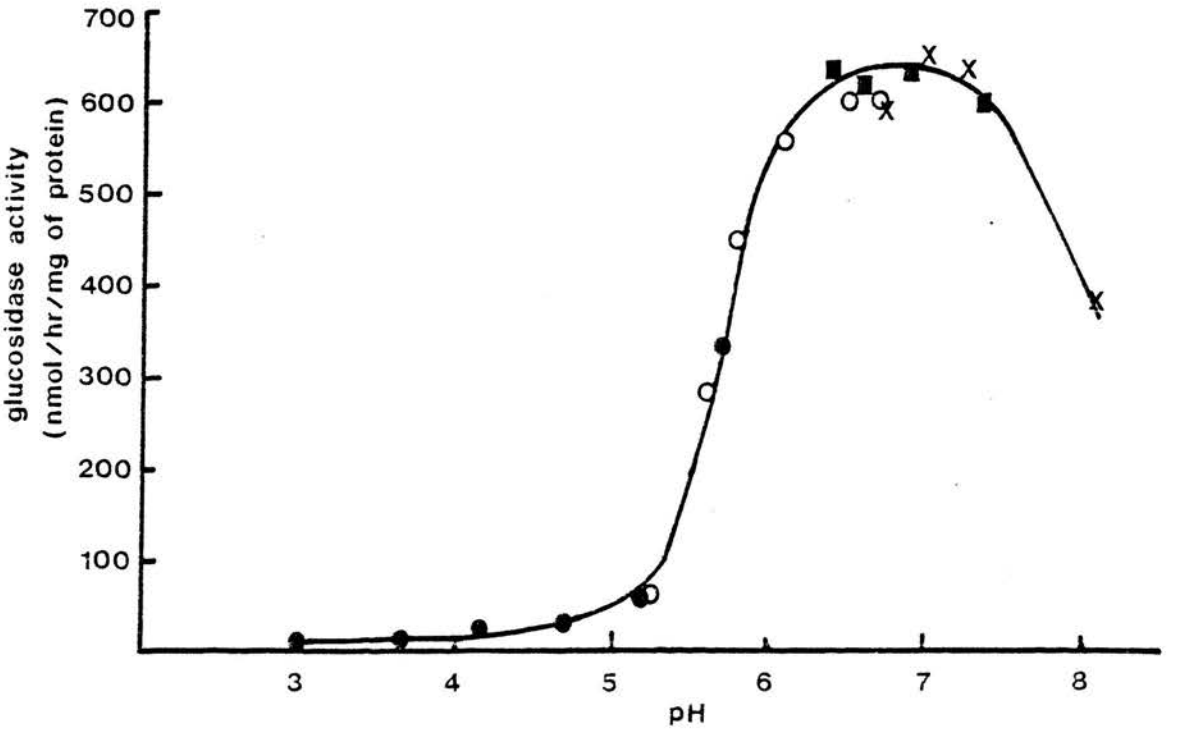



Figure 3.7d



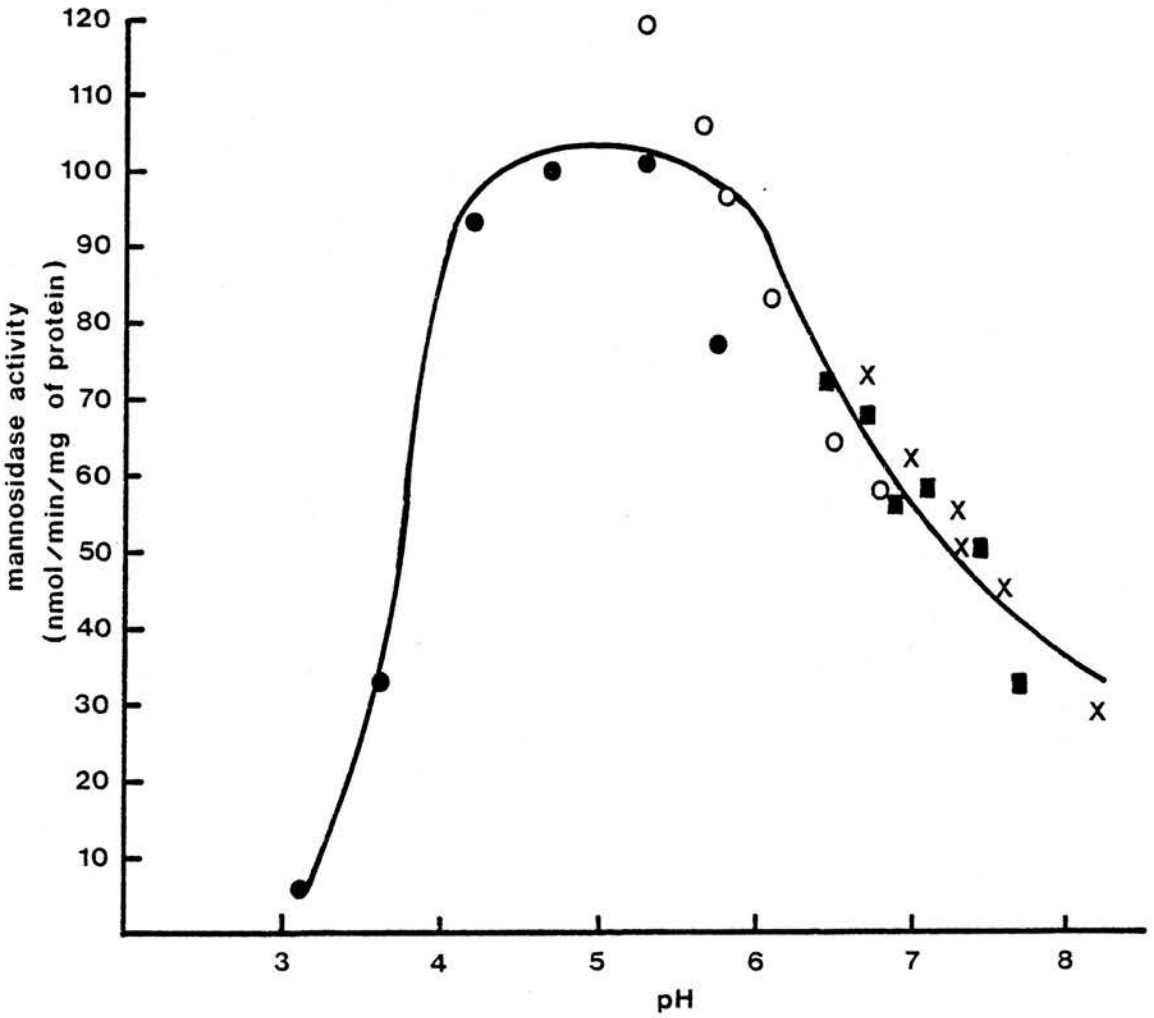
endoplasmic reticulum. The purified rat liver enzyme has a Km of 0.85mM, of the same order as that measured for the adrenal medullary microsomal enzyme; its Vmax is around 1000 times higher, 0.09μmol/hr/μg protein, than the adrenal microsomal enzyme (Michael and Kornfeld, 1980; Burns and Touster, 1982). Like the rat liver enzyme (Burns and Touster, 1982) the adrenal medullary enzyme was sensitive to inhibition by Tris HCl (50% at 20mM). Turanose, an effective inhibitor of lysosomal α-glucosidase at 1-5mM, had no effect on the adrenal medullary enzyme.

b. α-Mannosidase.

An α-mannosidase activity has been shown to be uniquely localised in rat liver endoplasmic reticulum (Bischoff and Kornfeld, 1983). It is distinct from α-mannosidases I and II and lysosomal α-mannosidase. Golgi α-mannosidase I shows no activity towards pnp-α-mannoside, whereas the RER enzyme does. If present in appreciable amounts in adrenal medullary RER fractions this enzyme could be used as a marker.

α-mannosidase activity of adrenal medullary microsomes was proportional to time of incubation and the amount of protein added. The pH profile of adrenal medullary mannosidase activity was broad (Figure 3.8) suggesting that two or more activities were present. However, by assaying mannosidase activity at pH7.4 the contribution from lysosomal activity was minimised. At pH7.4 α-mannosidase had a Km of 0.24mM with a Vmax of 0.076nmol/hr/μg of protein. The dissociation constant is almost identical to that estimated for the enzyme purified from rat liver. Unfortunately there was a large contribution to the total activity from acid-α-mannosidase, it was unlikely that this minor activity would be a useful marker; however, the distribution of mannosidase activities at pH5.5 and 7.4 was

Figure 3.8. pH-Profile of Microsomal Membrane -Mannosidase Activity.




Microsomal membranes (0.34mg of protein/ml) were incubated at the given pH for 2hr in 50mM of the following buffers: citrate (pH3-5.4); Mes (pH5.5-6.8); Mops (pH6.5-7.8); Hepes(pH6.8-8.2). Unlike the membranes used for the glucosidase assay these were not washed in buffer prior to assay. The reaction was quenched with $Na_2CO_3$ and the release of p-nitrophenol estimated spectrophotometrically. A parallel control incubation, without substrate, was quenched after 2hr at $37^oC$ with $Na_2CO_3$ containing pnp-mannoside.

investigated (see below).

Distribution of Marker Enzymes on Sucrose Gradients.

Figure 3.9 shows a fractionation of adrenal medullary microsomes on a continuous gradient of sucrose. In addition to the marker enzymes described earlier the two glycosidases described above and dopamine $\beta$-hydroxylase were used. Protein was distributed between two peaks with buoyant densities in sucrose of 1.123g/ml and 1.175g/ml; galactosyltransferase activity was associated with the former, while RNA was associated with the latter. RNA was however distributed throughout the gradient. It is interesting that the glucosidase activity does not equilibrate with the $\alpha$-mannosidase activity measured at pH7.4. This activity appears to sediment between the glucosidase and galactosyltransferase activity suggesting that this activity may be marking the medial Golgi membranes, at this pH $\alpha$-mannosidase II which shows activity to pnp-mannoside, will be separated from lysosomal activity at pH5.5 marking the distribution of lysosomal membrames.

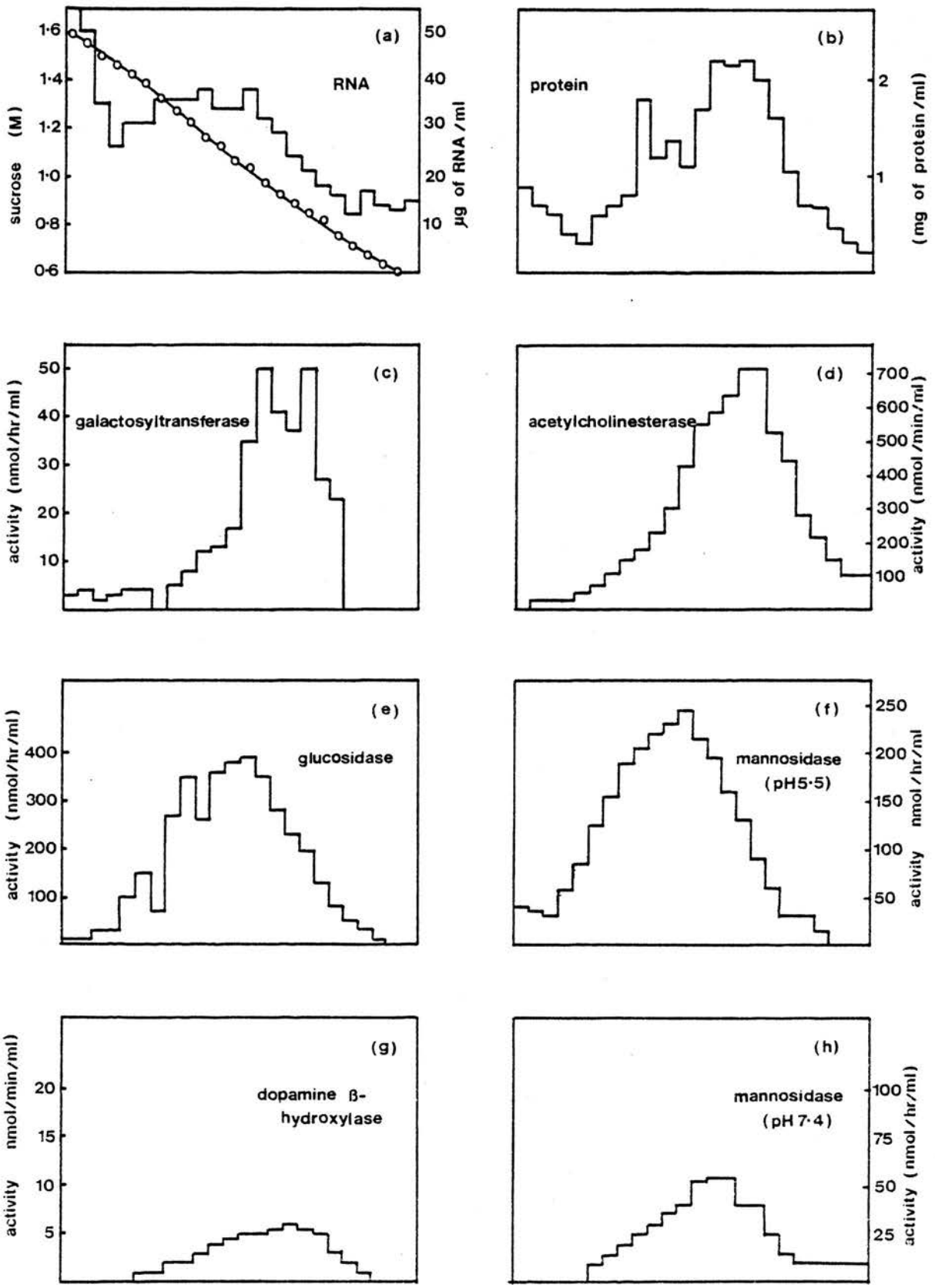
In this particular experiment galactosyltransferase activity was resolved into two peaks. This may reflect the distibution of complete Golgi stacks and trans elements derived from them during homogenisation. Acetylcholinesterase activity does not equilibrate with the peak of Galactosyltransferase activity. At a density of 1.090g/ml it has a lower buoyant density than has been estimated for plasma membrane by other workers. The specific activity for DBH estimated for these fractions was 30nmol/min/mg of protein. This suggests that when compared to pure chromaffin granule membranes (Figure 3.10) 5.6% of the microsomal membranes may be chromaffin granule membranes; a similar value (6.8%) has been reported by

Figure 3.9. Distribution of Marker Enzymes After Isopycnic Centrifugation of Adrenal Medullary Microsomal Membranes.

Microsomal membranes were recovered from a 1.4M-sucrose cushion (see preparative methods Chapter 2) and then washed in buffered 0.3M-sucrose. After centrifugation at 161,000g$_{av}$ for 60min the membrane pellets were resuspended in buffered 0.3M-sucrose then adjusted to 1.6M-sucrose and 0.5ml samples layered under six continuous gradients ofsucrose (0.5-1.6M). After centrifugation for 15hr at 196,000g$_{av}$ in an SW41Ti rotor the gradients were fractionated and samples combined, diluted 3-4-fold with buffer and membranes recovered by centrifugation at 171,000g$_{av}$ in a 70.1Ti rotor. Samples were assayed for: a. RNA, b. Protein, c. galactosyltransferase, d. acetylcholinesterase, e. $\alpha$-glucosidase, f. $\alpha$-mannosidase (pH5.5), g. dopamine $\beta$-hydroxylase, and h. $\alpha$-mannosidase (pH7.4).

Of the total protein loaded onto the gradients 71% was recovered with the membranes. Glycosidase activities were assayed in this experiment by incubation at 37$^{o}$C for 120min.

Figure 3.9


(a)
RNA
sucrose (M)
1·6
1·4
1·2
1·0
0·8
0·6
50
40
30
20
10
µg of RNA/ml
(b)
protein
2
1
(mg of protein/ml)
(c)
galactosyltransferase
activity (nmol/hr/ml)
50
40
30
20
10
(d)
acetylcholinesterase
700
600
500
400
300
200
100
activity (nmol/min/ml)
(e)
glucosidase
activity (nmol/hr/ml)
400
300
200
100
(f)
mannosidase
(pH5·5)
250
200
150
100
50
activity nmol/hr/ml
(g)
dopamine ß-
hydroxylase
activity nmol/min/ml
20
15
10
5
(h)
mannosidase
(pH 7·4)
100
75
50
25
activity (nmol/hr/ml)

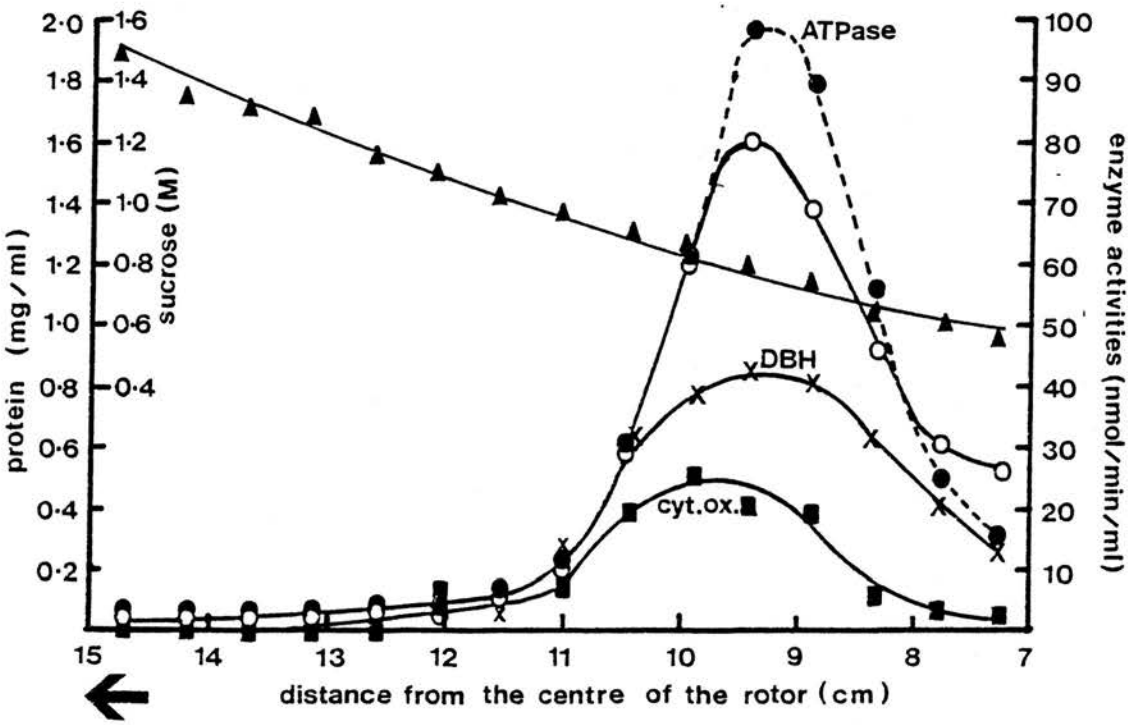
Corcoran et al., (1982). However there is no peak of DBH activity at the density at which chromaffin granule membranes peak (1.100g/ml), suggesting that the DBH activity may be due to either soluble DBH associated with the

microsomal membranes or, that this is indeed an endogenous microsomal activity. The content of cytochrome $b_{561}$ in the microsomal membranes would suggest however, that these membranes may be contaminated by up to 14% with chromaffin granule membranes.

Purification of Chromaffin Granule Membranes.

Chromaffin granule membranes were further purified by isopycnic centrifugation on continuous gradients of sucrose (Figure 3.10). A broad band of protein was recovered at a density of 1.100g/ml. Dopamine $\beta$-hydroxylase activity was distributed across this band of protein, the peak fraction having a specific activity for DBH of 532nmol/min/mg of protein. Purified mitochondrial inner membrane equilibrated on these sucrose gradients with a buoyant density of 1.189g/ml; with cytochrome oxidase as the marker enzyme (Figure 3.11). However a minor peak of cytochrome oxidase activity was associated with purified chromaffin granule membranes. This activity did not equilibrate however with either the protein peak or the marker enzymes for chromaffin granule membranes (see Figure 3.10). Thus there is a population of mitochondrial inner membrane fragments co-sedimenting with chromaffin granule membranes purified on step gradients of sucrose. This activity may be physically associated with fragments of the outer membranes of mitochondria which are known to co-sediment with Golgi membrane fractions.

Figure 3.10. Isopycnic Centrifugation of Purified Chromaffin Granule Membranes.

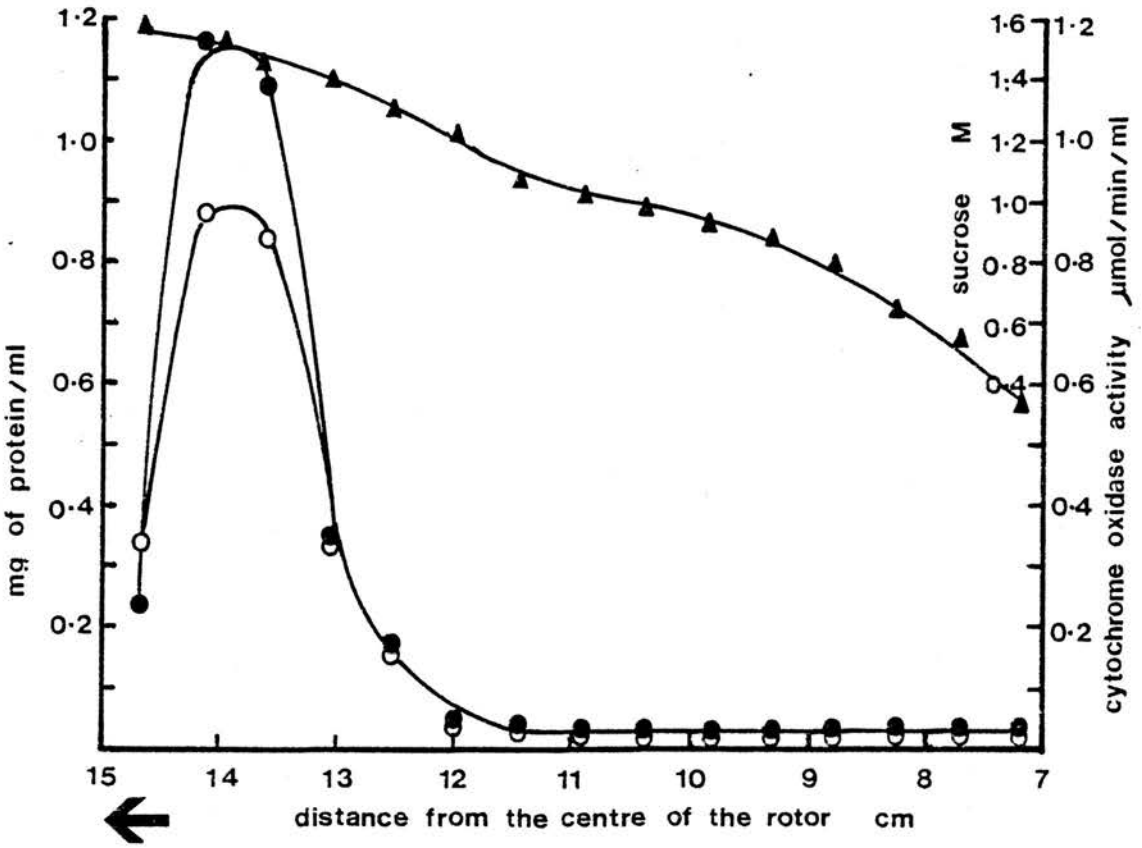



Chromaffin granule membranes (Fraction 9, Figure 3.1) were resuspended in 1.6M-sucrose and 0.5ml fractions (containing 1.5mg of protein) were layered under four continuous gradient of sucrose (0.5M-1.4M). Fractions were combined and diluted with buffer then recovered by centrifugation for 60min at 171,000$g_{av}$ in a 70.1Ti rotor. Fractions were resuspended in 0.5ml of buffer.

Fractions were assayed for -O-O- protein, -■-■- cytochrome oxidase, -×-×- dopamine -hydroxylase and -●-●- ATPase activity under the assay conditions described in analytical methods Chapter 2.

Protein recovered 62% of load sample. ATPase activity recovered 32%.

Figure 3.11. Isopycnic Centrifugation of Purified Mitochondrial Membranes.




0.5ml of mitochodrial membranes (4mg/ml) were layered on top of a continuous gradient of sucrose (0.5M-1.6M) and centrifuged at 196,000$g_{av}$ for 16hr in an SW41Ti rotor. After the gradient membrane fractions had been washed in buffer they were assayed for: ●—● cytochrome oxidase activity; O—O protein and ▲—▲ sucrose.

a. ATPases.

The chromaffin granule membrane fractions exhibit two $Mg^{2+}$ dependent ATPase activities (Apps et al., 1983). These activities equilibrated as a single peak with DBH (Figure 3.10). Ouabain-sensitive $Na^+,K^+$-ATPase is a marker for plasma membrane, but in the adrenal medulla very little ATPase activity proves to be sensitive to ouabain. It is now known that many other cellular membranes contain ATPases, distinguishable to some extent by their sensitivity to inhibitors. ATPase measurements were made on some fractions (Table 3.4).

The inhibitor sensitivity of the ATPase activity in the Golgi membrane enriched fraction (microsomal band II) was compared with chromaffin granule membrane ATPase (Table 3.5). Although there was much similarity, the Golgi enzyme failed to show the inhibition by DCCD and NEM that are characteristic of the proton-translocating ATPase of chromaffin granules.

b. Cytochrome $b_{561}$.

Cytochrome $b_{561}$ has been identified in microsomal fractions from adrenal medullary tissue (Spiro and Ball, 1958; Ichikawa and Yamano, 1965; Flatmark et al., 1971), with only traces having been identified in endoplasmic reticulum fractions (Hörtnagl, 1976). There was no enrichment of cytochrome $b_{561}$ in any of the microsomal fractions (Table 3.3). Spectrophotometric analysis required large amounts of membrane making routine analysis difficult. Figure 3.12 shows the spectra obtained for the three microsomal fractions. All three spectra are identical.

Table 3.4. ATPase Activity of Adrenal Medullary Microsomal Membranes.

| Fraction | ATPase Activity. nmol/min/mg. |
|---|---|
| Microsomes | 118 |
| Band I | 24 |
| Band II | 239 |
| Band III | 154 |
| CGM | 242 |

Table 3.5. Effects of ATPase Inhibitors on Golgi Membrane ATPase.

| Inhibitor | Chromaffin Granule | | Golgi Membrane | |
|---|---|---|---|---|
| | [Inhib.] | % Act. | [Inhib.] | % Act. |
| Antimycin | | | 10μM | 100 |
| Azide, Sodium | 3.6mM | 100 | 1mM | 90 |
| Nbf-Cl | 10μM | 23 | 100μM | 50 |
| Nbf-Cl + DTT | ND | - | 100μM | 100 |
| DCCD | 100μM | 42 | 100μM | 100 |
| Efrapeptin | ND | - | 20μg/ml | 100 |
| Ethanol | 1% | 100 | 1% | 100 |
| Fluoride, $K^+$ | ND | - | 4mM | 100 |
| N-ethylmalaimide | 100μM | 16 | 100μM | 83 |
| Oligomycin | ND | - | 50μg/ml | 85 |
| Orthovanadate, $Na^+$ | 10μM | 82 | 10μM | 100 |
| Quercetin | 66μM | 62 | 66μM | 37 |
| TBT-$Cl^-$ | 10μM | 31 | 20μM | 32 |
| Triton X-100 | ND | - | 0.02% | 185 |

Inhibition is shown as the percentage of the activity remaining, of the two fractions shown in Table 3.4. Golgi membranes were present at 27μg/ml of protein, chromaffin granule membranes at 25μg/ml.

FIGURE 3.12. Absorption Spectra of the Microsomal Membrane Fractions.

Microsomal membrane fractions (6,7&8, Figure 3.1) and chromaffin granule membranes were dialysed against 0.3M-sucrose 20mM-Hepes NaOH, pH7.2; 0.5mM-DTT; 1mM-EDTA to remove catecholamines before measuring their reduced minus oxidised difference spectra.

(a). Chromaffin granule membranes (0.25mg of protein/ml). Specific cytochrome content 4.61 nmol/mg of protein.

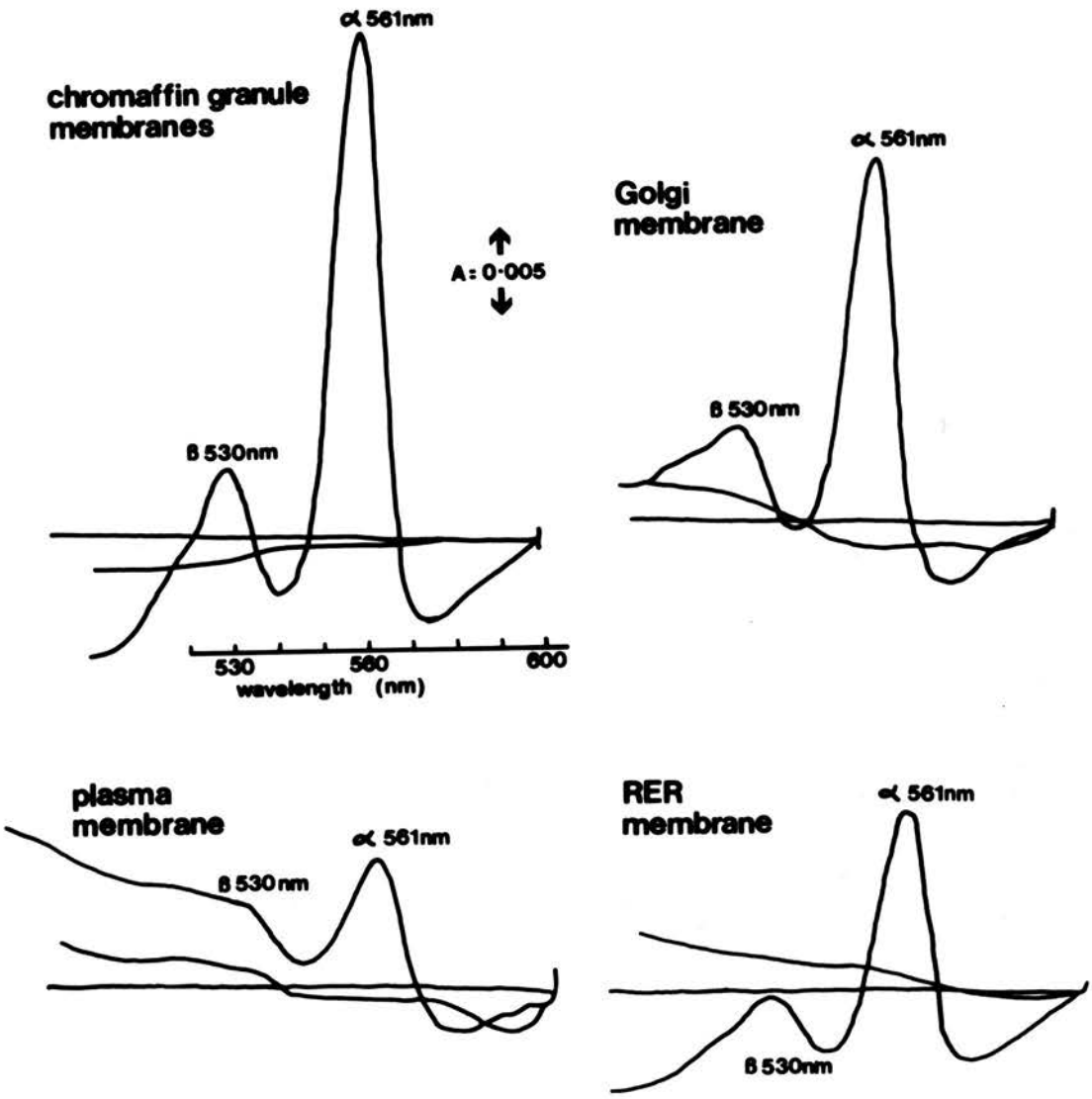
(b). Plasma membrane (Fraction 6) (0.53mg of protein/ml). Specific cytochrome content 0.63nmol/mg of protein.

(c). Golgi-enriched membranes (Fraction 7) (1.16mg of protein/ml). Specific cytochrome content 1.22nmol/mg of protein.

(d). RER-enriched membranes (Fraction 8) (1.48mg of protein/ml). Specific cytochrome content 0.56nmol/mg of protein.

The absorption maxima for the cytochrome $b_{561}$ are: an $\alpha$-peak at 561nm, a $\beta$-peak at 530nm and a large Soret-peak at 428nm (the latter is not shown).

Figure 3.12


chromaffin granule membranes
α 561nm
β 530nm
A: 0·005
530
560
600
wavelength (nm)
Golgi membrane
α 561nm
β 530nm
plasma membrane
α 561nm
β 530nm
RER membrane
α 561nm
β 530nm

Adrenocortical Contamination.

Adrenal medullary tissue is intimately associated with adrenocortical tissue. How much of this tissue contaminates adrenal medullary preparations?

To estimate this contamination fractions were assayed for their cytochrome P450 content (Omura and Sato, 1964), a marker for adrenocortical microsomal membranes; and were shown to have a cytochrome P450 activity of 567pmol/mg of protein. Adrenal medullary microsomal fractions were therefore apparently contaminated with about 10% adrenocortical membranes. This may be an overestimate however, as adrenal medullary tissue may have an endogenous cytochrome p450 content; there may also be a contribution from adrenocortical mitochondrial membranes.

Discussion.

Golgi-enriched microsomes have been isolated from bovine adrenal medullary tissue (Trifaró and Duerr, 1976) and from another endocrine tissue secreting hormones, the porcine anterior hypophysis (Jacobs et al., 1973; Mckeel and Jarett, 1974). A Golgi membrane enriched fraction was isolated in this study 6- to 8-fold enriched in the marker enzyme for Golgi membranes galactosyltransferase. Another marker for the Golgi complex, mannosidase II also equilibrated with galactosyltransferase and appeared to be marking a slightly less buoyant fraction possibly cis- to medial-cisternal membranes. The Golgi membrane fraction may however be substantially contaminated by plasma membrane, identified by acetylcholinesterase. While it has been assumed that acetylcholinesterase is uniquely located in the adrenal medullary plasma membrane its distribution is complicated by both the soluble form of this enzyme redistributing during fractionation and its apparent presence in the chromaffin granule; however its distribution remains controversial (Burgun et al., 1985).

Microsomal membrane Band I is thought to be enriched in plasma membrane in view of (i) its enrichment in acetylcholinesterase and the shift in the buoyant density of this enzyme to lower density after osmotic lysis of the microsomal fraction (ii) the enrichment in 5'-nucleotidase in this fraction. The buoyant density of this fraction is lower than that reported for previous preparations, but this fraction, although its yield is low, does appear to be purer than the previous preparations of Wilson and Kirshner (1976). Further evidence for the plasma membrane nature of this fraction is provided by lectin binding experiments described in Chapter 4.

The Golgi membrane fraction isolated in this study is enriched

in galactosyltransferase to the same extent as previously described for adrenal medullary tissue by Trifaró and co-workers (1976). However, in the present study enrichments were with respect to a post-nuclear fraction rather than to whole homogenate as described by Trifaró et al. (1976) and may by comparison be an underestimate.

This study confirms the heterogeneous nature of microsomal fractions from adrenal medullary tissue. Fractionation based on buoyant density does not discriminate adequately between different subcellular compartments, particularly if those of interest are not very abundant. Furthermore, some compartments may not necessarily be either morphologically or biochemically distinct from each other.

That the reticular membrane system can be fractionated at all after homogenisation is remarkable. By its very nature it is also biochemically heterogeneous and therefore fractions produced by centrifugation procedures on sucrose gradients tend to overlap; the buoyant densities of these fractions are governed by their lipid content which increases from the RER to plasma membrane.

The way forward is to raise antibodies to cytoplasmically oriented epitopes of integral membrane proteins of the endomembrane compartments, to enable affinity purification procedures to be exploited. Removal of plasma membrane from fractions by this approach with antibodies raised to cell surface proteins would greatly simplify subsequent fractionation. Such an approach naturally depends upon identification of proteins unique to these locations and highlights the need for an electrophoretic characterisation of RER and Golgi-membrane proteins in conjuction with immunolocalisation studies at the resolution of the electron microscope. The first tentitive steps towards the former in adrenal medullary tissue are described in Chapter 4.

# CHAPTER FOUR

# ELECTROPHORETIC ANALYSIS OF ADRENAL MEDULLARY SUBCELLULAR FRACTIONS.

Introduction.

Having isolated fractions enriched in RER, Golgi and plasma membranes in addition to previously characterised chromaffin granule membrane and mitochondrial membrane fractions, it was now possible to try to answer some of the questions posed in the introduction to Chapter 3, in particular whether biogenic precursor polypeptides of cytochrome $b_{561}$ and DBH could be identified in these fractions using antibodies to the mature forms of these proteins.

While there have been numerous detailed electrophoretic studies on the chromaffin granule membrane, one of the more highly resolved of these being presented in Chapter 5, there has been comparatively little electrophoretic analysis of the endomembrane system involved in the intracellular transport and biogenesis of secretory proteins. The few analyses that have been published provide little comparative information other than that the chromaffin granule membrane appears to have a unique and relatively simple polypeptide composition. A finding common to many secretory granules is that they contain a relatively small number of major secreted and membrane polypeptides, other polypeptides being present at around 1-2% of the total granule protein, while the membranes in which they are synthesised and assembled appear to have more complex polypeptide patterns.

Subcellular fractions of adrenal medullary tissue have been analysed by one-dimensional electrophoresis (Winkler et al., 1970; Kirshner, 1974; Hörtnagl et al., 1971,1972; Helle, 1971; Hörtnagl, 1976; Wilson and Kirshner, 1976; Zinder et al., 1978). The electrophoretic pattern of chromaffin granule membrane proteins is very different from that of the total microsomal membrane fraction (Winkler et al., 1970).

Zymogen granule membranes also show distinct differences when compared with their RER and mitochondrial membranes, although some similarities with plasma membrane polypeptide patterns have been found (Meldolesi and Cova, 1972). These studies however lacked the degree of resolution which is now possible with modern one-dimensional gel systems, and no comparisons of more than two or at most three different membrane fractions have been made. By analysing the purified microsomal membrane fractions enriched in markers for the RER, Golgi and plasma membranes, and comparing them with the membrane proteins of the chromaffin granule it may be possible to uncover the biosynthetic relationship between these endomembrane compartments. For example, does each compartment have a distinct set of proteins which may reflect its function? Do compartments share common proteins, for example structural proteins or proteins with a general function in transport and sorting?

## One-Dimensional Analysis.

The relative simplicity of the chromaffin granule membrane polypeptide composition after staining one-dimensional polyacrylamide gels with Coomassie blue is shown in track 5 of Figure 4.1; the major proteins are indicated. The mitochondrial membranes (Track 2) like chromaffin granule membranes, also have a unique polypeptide composition, the major polypeptides being the catalytic subunits of the $F_1F_0$-ATPase ($M_r$50-52,000). Microsomal membranes (Track 6) are dominated by a polypeptide of $M_r$68,000 and even after fractionation the complexity of this fraction is still reflected in the membrane fractions enriched in Golgi membranes (Fraction 7; tracks 4&8) and RER-membranes (Fraction 8; tracks 3&9).

However, these fractions do show some striking differences in their polypeptide patterns, in particular, the polypeptide of $M_r$68,000 is enriched in the Golgi membranes, as are a number of other polypeptides. While the heterogeneity of microsomal fractions 7&8 may reflect their intimate biosynthetic relationship it must also certainly reflect the inherent heterogeneity of the microsomal fractions a product of homogenisation. These fractions have been isolated under conditions which should retain their vesicular nature and thus they will retain their soluble luminal proteins. The plasma membrane enriched fraction (Fraction 6; track 7) is particularly striking being enriched in a protein of $M_r$32,000. Of significance is the presence of cytochrome $b_{561}$ in all the microsomal membrane fractions in which their is comparatively little DBH and chromogranin A.

**Figure 4.1. One-Dimensional Electrophoretogram of Membrane Fractions Isolated From Adrenal Medullary Tissue.**

This figure is a composite of two 7-15% gels of membrane fractions isolated as shown in Figure 3.1. Samples were precipitated with acetone ethanol (see electrophoretic methods Chapter 2) solubilised in sample buffer and reduced with 5% mercaptoethanol. The gel is stained with Coomassie blue.

Track 1, standard proteins: β-galactosidase ($M_r$ 130,000); phosphorylase b ($M_r$ 94,000); bovine serum albumin ($M_r$ 68,000); ovalbumin ($M_r$ 43,000); carbonic anhydrase ($M_r$ 30,000); trypsin inhibitor ($M_r$ 21,000), myoglobin ($M_r$ 17,000) lysozyme ($M_r$ 14,300).

Track 2, mitochondrial membranes (100µg of protein from the peak fraction of the continuous gradient in Figure 3.11).

Track 3&9, RER-enriched membranes (Fraction 8 of Figure 3.1).

Track 4&8, Golgi-enriched membranes 100µg of protein (Fraction 7, Figure 3.1).

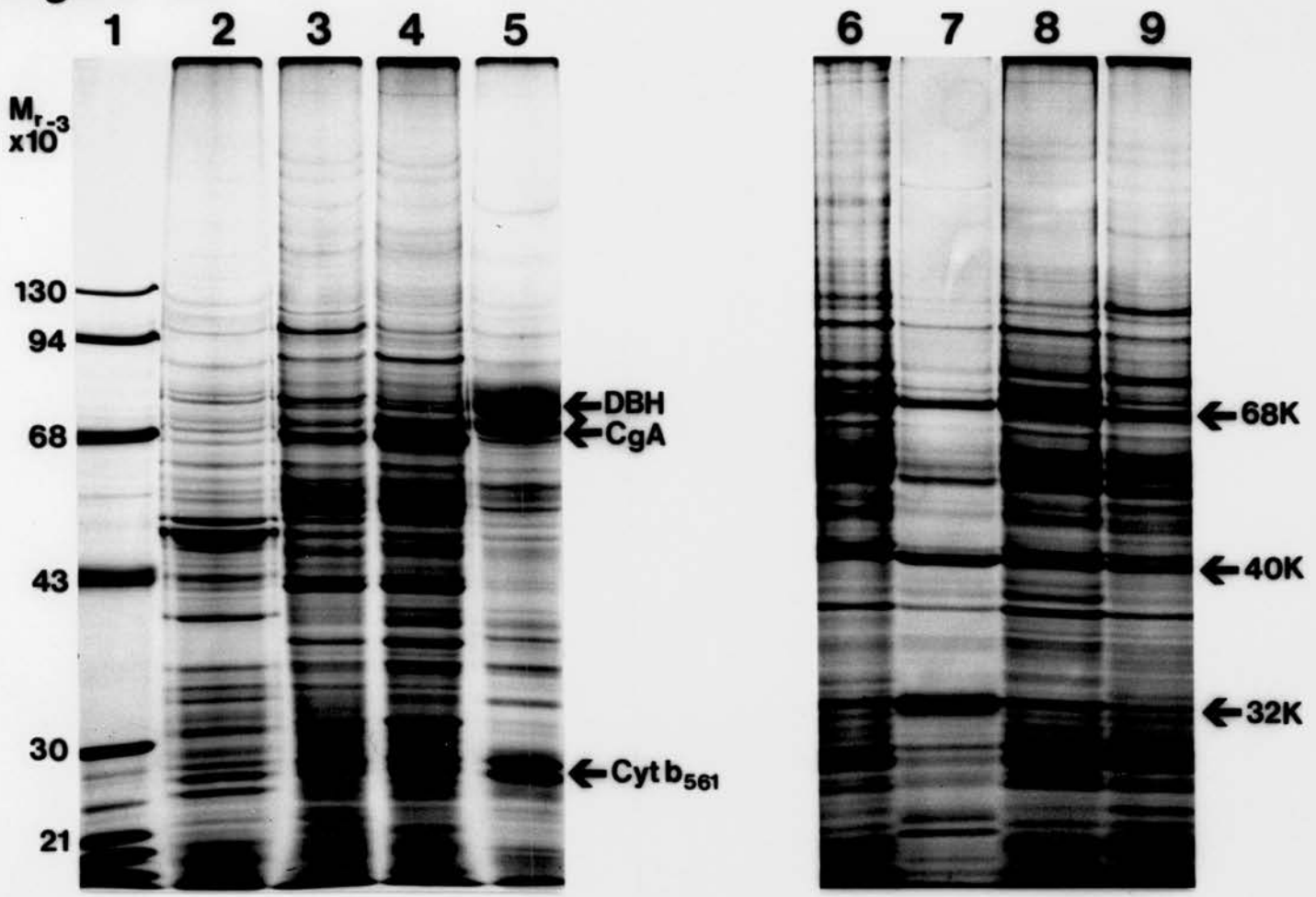
Track 5, chromaffin granule membranes 100µg of protein (Fraction 9 Figure 3.1).

Track 6, total microsomal membranes 100µg of protein (Fraction 5, Figure 3.1).

Track 7, plasma-membrane enriched fraction 100µg of protein (Fraction 6, Figure 3.1).

The major proteins of the chromaffin granule membrane are indicated; chromogranin A, CgA; cytochrome $b_{561}$, Cyt$b_{561}$ and dopamine β-hydroxylase, DBH.

**Figure 4.1.**


1
2
3
4
5
6
7
8
9
$M_r$ -3 x10
130
94
68
43
30
21
DBH
CgA
Cyt $b_{561}$
68K
40K
32K

**Washing Membranes With Sodium Carbonate.**

A major problem when analysing membrane fractions are adhering soluble cytoplasmic and luminal proteins (this is discussed in Chapter 5). Because the microsomal fractions have been isolated here in a way that retains their luminal protein content, these will tend to dominate protein maps on electrophoretograms. Chromaffin granules have a small membrane to luminal volume ratio and when subjected to hypotonic lysis they released 83% of the protein content. This figure agrees with previously published estimates (Hillarp, 1959; Winkler et al., 1970). The microsomal fractions enriched in plasma-, Golgi- and RER-membranes released respectively 34%, 57%, and 48% of their soluble proteins on hypotonic lysis in buffer. A more efficient removal of soluble proteins can be obtained however by washing membranes with $Na_2CO_3$ (Higgins, 1984; Howell and Palade, 1982)and respectively 32%, 64% and 78% of the soluble membrane associated proteins were released by this treatment. Figure 4.2a shows a one-dimensional electrophoretic analysis of the microsomal membrane fractions following washing with sodium carbonate and the soluble proteins washed from them are shown in Figure 4.2b. The gels also contain a track of granule lysate, showing the major secreted proteins of this tissue. Following this high-pH wash the differences in membrane polypeptide composition are more striking, although there are still many proteins common to all three microsomal fractions. For example a major protein of $M_r$40,000, and cytochrome $b_{561}$ ($M_r$24,000-28,000) are apparently present in each fraction to a greater or lesser degree. The soluble proteins associated with the Golgi-enriched membranes are dominated by a protein $M_r$68,000 which is distinct from chromogranin A $M_r$70,000, as shown by two dimensional analysis (Figure 4.8). There

**Figure 4.2. One-Dimensional Electrophoretograms of Adrenal Medullary Microsomal Membrane Fractions Washed With Sodium Carbonate.**

Microsomal membrane fractions were washed in $Na_2CO_3$ as described in the preparative methods section of Chapter 2 and analysed on 7-15% gels under reducing conditions.

**(a). $Na_2CO_3$ Washed Membrane Fractions.**

Track 1, standard proteins (as shown in Figure 4.1); track 2, plasma membranes; track 3, Golgi-enriched membranes; track 4, RER-enriched membranes; track 5, chromaffin granule membranes; track 6, chromaffin granule lysate proteins (40µg).

Each track contains 100µg of membrane protein.

**(b). Proteins Solubilised by Sodium Carbonate.**

Soluble proteins associated with fractions enriched in: track 2, plasma membrane; track 3, Golgi membrane (100µg Of protein); track 4, RER membrane (100µg of protein). Tracks 5&6, chromaffin granule secretory proteins (20µg and 100µg of protein).

**Figure 4.3. Solubilisation and Phase separation of Microsomal Membrane Fractions in Triton X-114.**

For the details of this figure consult Figure 5.4a, a phase separation of chromaffin granule membranes, with which this figure should be compared: (a) Golgi-enriched microsomal membranes; (b) RER-enriched microsomal membrane; (c) plasma membrane enriched microsomal fraction. Tracks: 1, standard proteins (see Figure 4.1); 2, whole fraction; 3, phospholipid-rich phase; 4, detergent-rich phase; 5, aqueous phase; 6, whole chromaffin granule membranes.

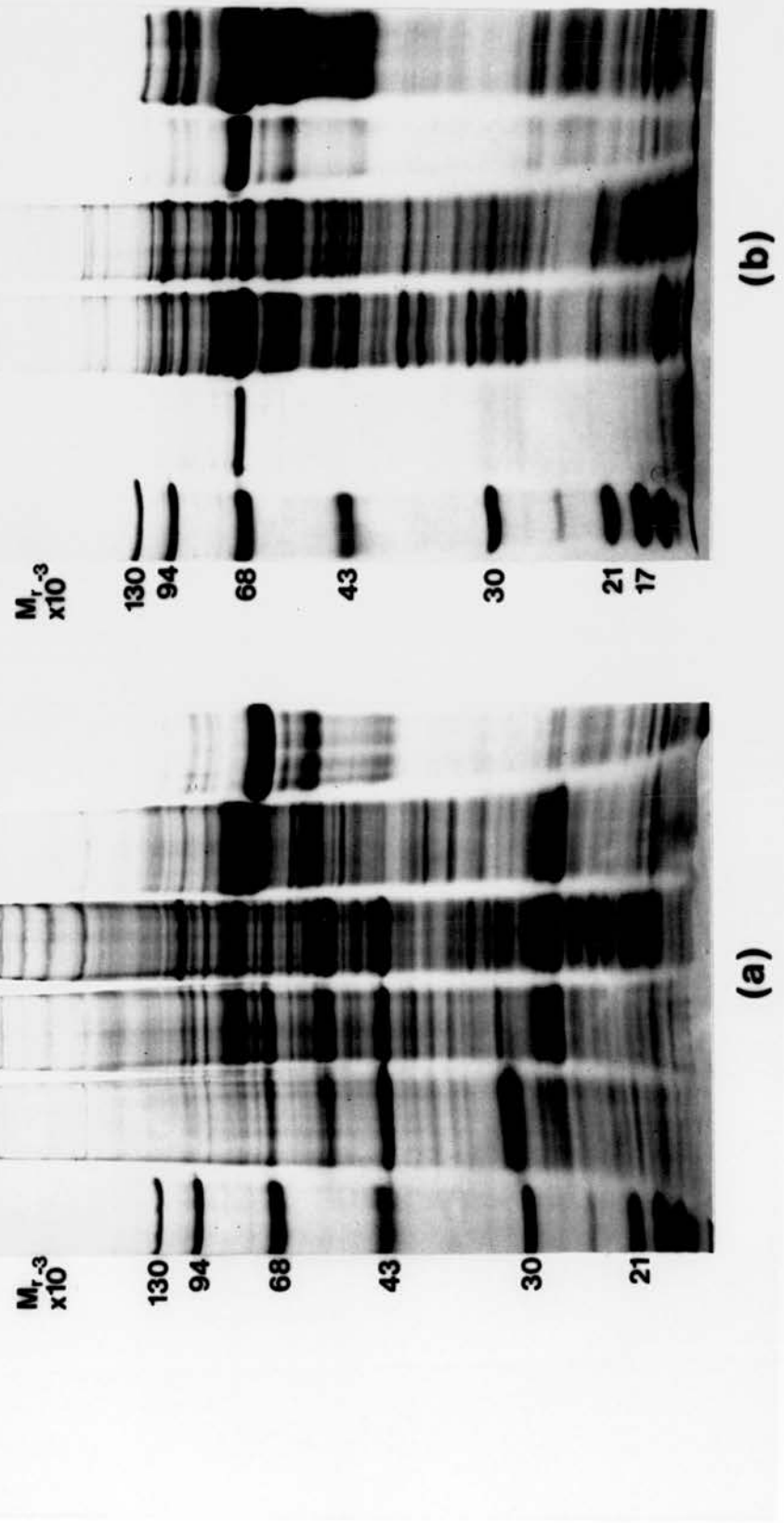
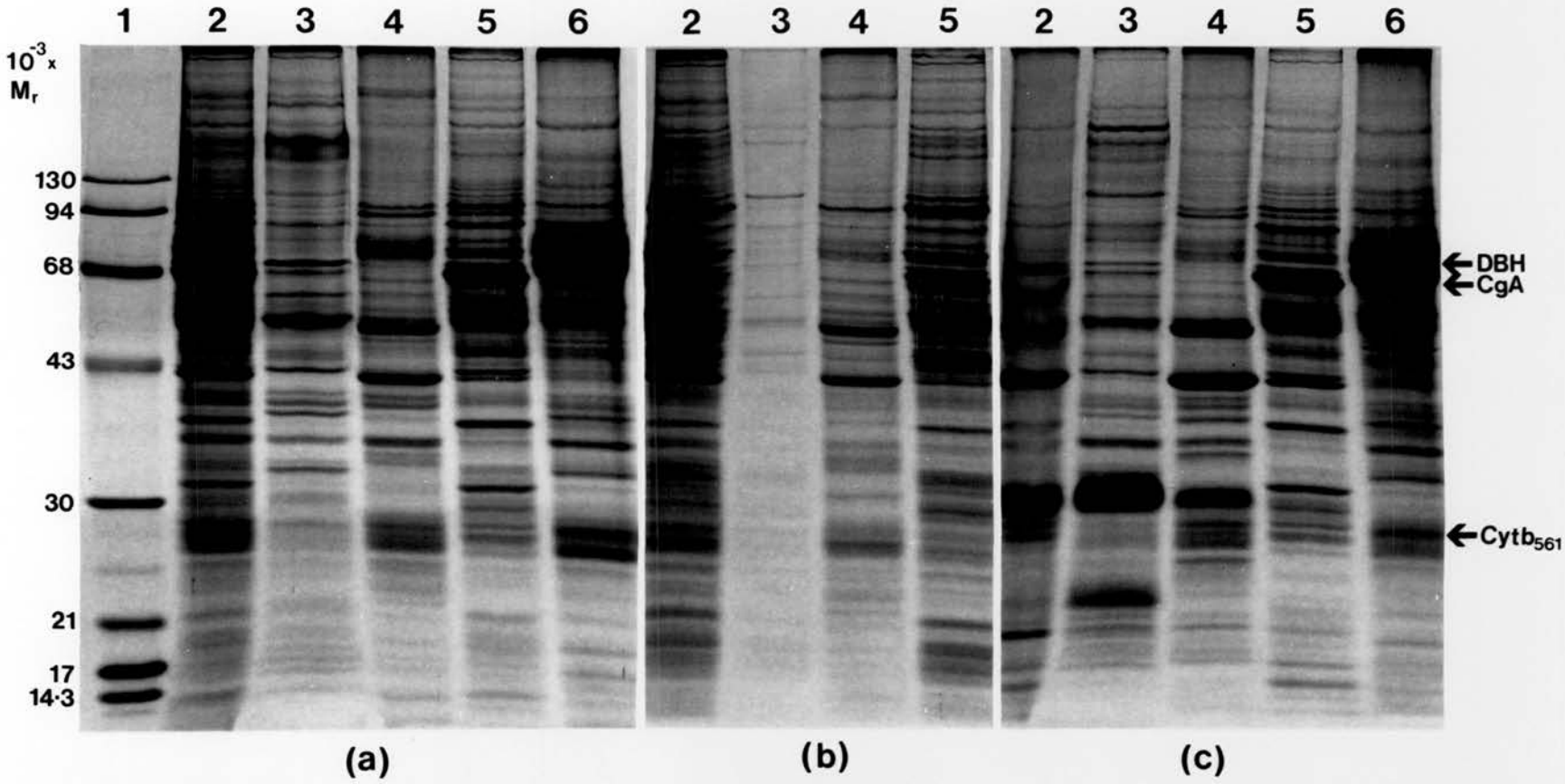



Figure 4.2.

Figure 4.3


1
2
3
4
5
6
2
3
4
5
2
3
4
5
6
$10^{-3}$ x
$M_r$
130
94
68
43
30
21
17
14·3
DBH
CgA
$Cytb_{561}$
(a)
(b)
(c)

considerable similarities between the polypeptides released from RER-enriched membranes and Golgi-enriched membranes. There are differences, however, and this combined with the fact that the galactosyltransferase activity is totally latent in these fractions suggests that these are luminal content proteins and not simply adsorbed cytosolic proteins. The small amount of soluble protein associated with the plasma membrane fraction may be indicative of much of this fraction consisting of broken membranes and/or flattened vesicles; hyposmotic lysis appears to be sufficient for the release of the majority of this soluble protein, while in contrast an additional 30% of the RER-membrane associated proteins can be removed by $Na_2CO_3$.

**Solubilisation and Phase Separation of Membranes in Triton X-114.**

Further differences between the microsomal membrane fractions were demonstrated by solubilising and phase separating the membrane proteins in Triton X-114 (this procedure is described in Chapter 5). Briefly, a characteristic of phospholipid-rich membranes is that they form a precipitate enriched in these components and some membrane proteins when solubilised in Triton X-114. Strikingly, the RER-enriched membrane fraction forms no precipitate (Figure 4.3,c). This fraction is dominated by proteins which partition into the aqueous phase consistent with the large percentage of protein removed from the membranes by $Na_2CO_3$, whereas both plasma membrane and Golgi-enriched fractions produce precipitates when solubilised, containing unique and characteristic proteins.

That the plasma membrane and Golgi fractions apparently have identical soluble protein patterns (compare track 5 in Figure 4.3,a&b) may be explained by the presence of Golgi membranes in the

plasma membrane fraction as revealed by marker enzyme analysis in Chapter 3. There are however two major proteins that are enriched in the plasma membrane fraction, but not in the Golgi-enriched membrane fraction. Two major integral membrane proteins in the detergent phase ($M_r$40,000 and $M_r$50,000) are common to all three fractions, but do appear to be slightly enriched in Band I, the plasma membrane fraction.

The phospholipid-rich precipitate produced by the Golgi membranes is interesting in that many of these polypeptides can be identified in an equivalent fraction following solubilisation of chromaffin granules membranes (compare Figures 4.3a, track 3 and Figure 5.4a, track 3). However, while the protein of $M_r$70,000 is common to both the Golgi and chromaffin granule membrane fractions it is much reduced in the plasma membrane fraction (Figure 4.3d) at least suggesting that fraction 6 (Figure 3.1) contains very little contamination from chromaffin granule membranes. This protein is the catalytic subunit of the chromaffin granule proton-translocating ATPase (Percy and Apps, 1986). This electrophoretic analysis correlates with the low $Mg^{2+}$ATPase activity measured in this fraction (Chapter 3). The phospholipid-rich precipitate produced by the plasma membrane fraction is dominated by a protein of $M_r$32,000 (Figure 4.3c), only a small percentage of which is present in the detergent phase. This protein is unique to this low density membrane fraction and its plasma membrane origin was supported by density centrifugation of microsomal membranes on dextran gradients as described by Avruch and Wallach, (1971). The protein ($M_r$32,000) was identified, by lentil lectin overlay analysis (see below), with membranes banding at the very top of the gradient; this distribution on dextran in a medium of low ionic strength containing $Mg^{2+}$ is characteristic of many plasma membrane fractions.

Two-Dimensional Electrophoretic Analysis.

1. Soluble Proteins.

Figure 4.4 shows a two-dimensional electrophoretogram of the soluble chromaffin granule matrix proteins stained with Coomassie blue. The polypeptide pattern is dominated by the main secretory protein chromogranin A ($M_r$ 70,000, pI 4.8) and its proteolytic breakdown products. These have been identified using antisera to the parent protein. In addition two other families of acidic secretory proteins, chromogranins B and C, and their attendant proteolytic fragments have been identified (Fischer-Colbrie and Frishenschlager, 1985; Kilpatrick et al., 1983; Winkler et al., 1984; Apps et al., 1985). A small amount of the soluble form of DBH can also be identified. It is particularly striking that these proteins occupy a narrow window of the pH-gradient. While being well characterised electrophoretically the function of the majority of these proteins is unknown.

2. Chromaffin Granule Membrane Proteins.

Figure 4.5 shows a two-dimensional electrophoretogram of chromaffin granule membranes which have been subjected to repeated rounds of washing in hyposmotic buffer to remove soluble proteins. One of the major proteins appears to be chromogranin A, however, it can be further reduced by repeated washing in sodium carbonate. The major Coomassie staining integral membrane protein is cytochrome $b_{561}$ ($M_r$ 24-28,000, pI6.2; Apps et al., 1980a). DBH, the other major membrane protein, focuses poorly and fails to enter the second dimension gel efficiently. Another significant protein is glycoprotein III however like many of the glycoproteins of the membrane it stains poorly with Coomassie. An analysis of these proteins is presented in Chapter 5.

**Figure 4.4. Two-Dimensional Electrophoretogram of Chromaffin Granule Matrix Proteins.**

Chromaffin granule matrix proteins (120µg) were loaded at the cathodic end of the focusing gel in the presence of mercaptoethanol. After focusing proteins were separated in the second dimension in the presence of SDS then stained with Coomassie blue.

The major secretory proteins chromogranin A ($M_r$ 70,000; CgA) and chromogranin B ($M_r$ 100,000; CgB) are indicated. The majority of spots of lower $M_r$ and identical pI to CgA and CgB are proteolytic breakdown products of these two parent molecules.

The position of the soluble form of dopamine β-hydroxylase (sDBH) ($M_r$ 72,000; DBH) is shown.

All these polypeptide spots have been identified with antisera to the purified proteins (see Winkler et al., 1986; Fischer-Colbrie and Frischenschlager, 1985; Apps et al., 1985; Winkler et al., 1984; Kilpatrick et al., 1983.

**Figure 4.5. Two-Dimensional Electrophoretogram of Chromaffin Granule Membrane Proteins.**

Chromaffin granule membranes were washed six times in 10mM-Hepes NaOH, pH7.2 to remove soluble proteins. Membrane associated proteins (250µg) were analysed in the presence of 0.5% mercaptoethanol (no additional mercaptoethanol was present in the second dimension). Proteins were identified by immune blotting and lectin overlays (Winkler et al., 1986).

The positions of dopamine β-hydroxylase (DBH), cytochrome $b_{561}$ ($Cytb_{561}$) and glycoprotein III (GpIII) are indicated. Subunits of the proton translocating ATPase I are shown (1, $M_r$ 70,000 and 2, $M_r$ 53,000). Adhering soluble proteins include: chromogranin A (CgA; proteolysis products are marked by arrows), chromogranin B (CgB) and mitochondrial β-subunit of the $F_1$ ATPase ($F_1$).

Figure 4.4.

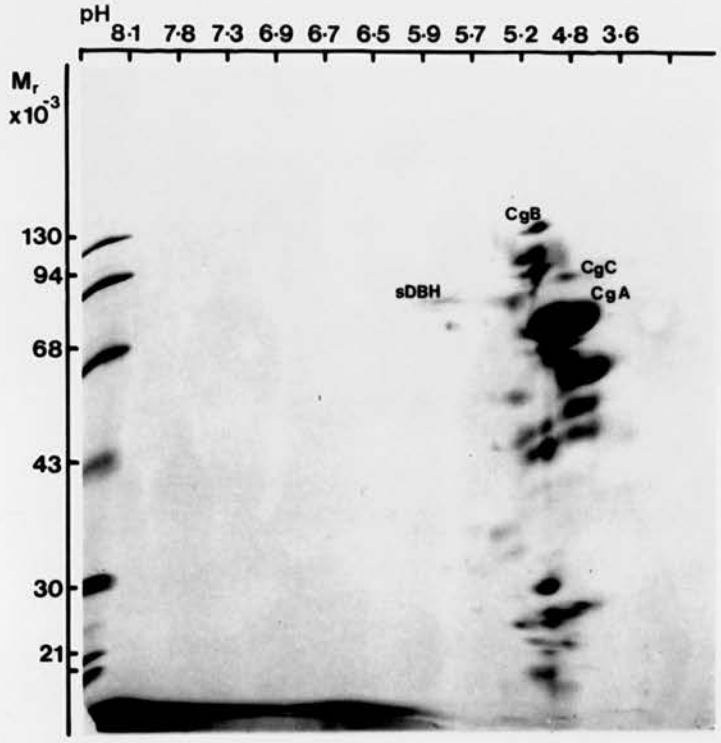
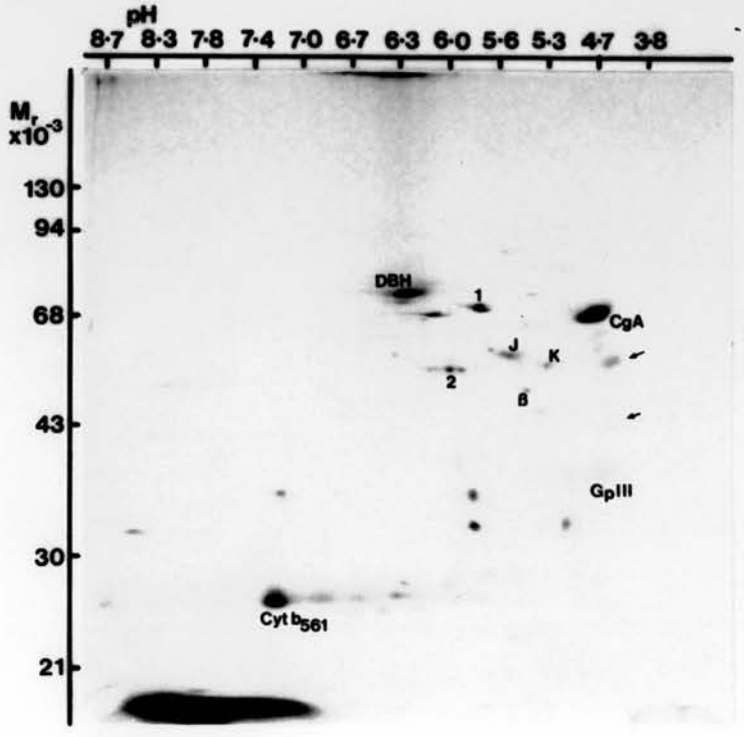



Figure 4.5.



### 3. Mitochondrial Membranes.

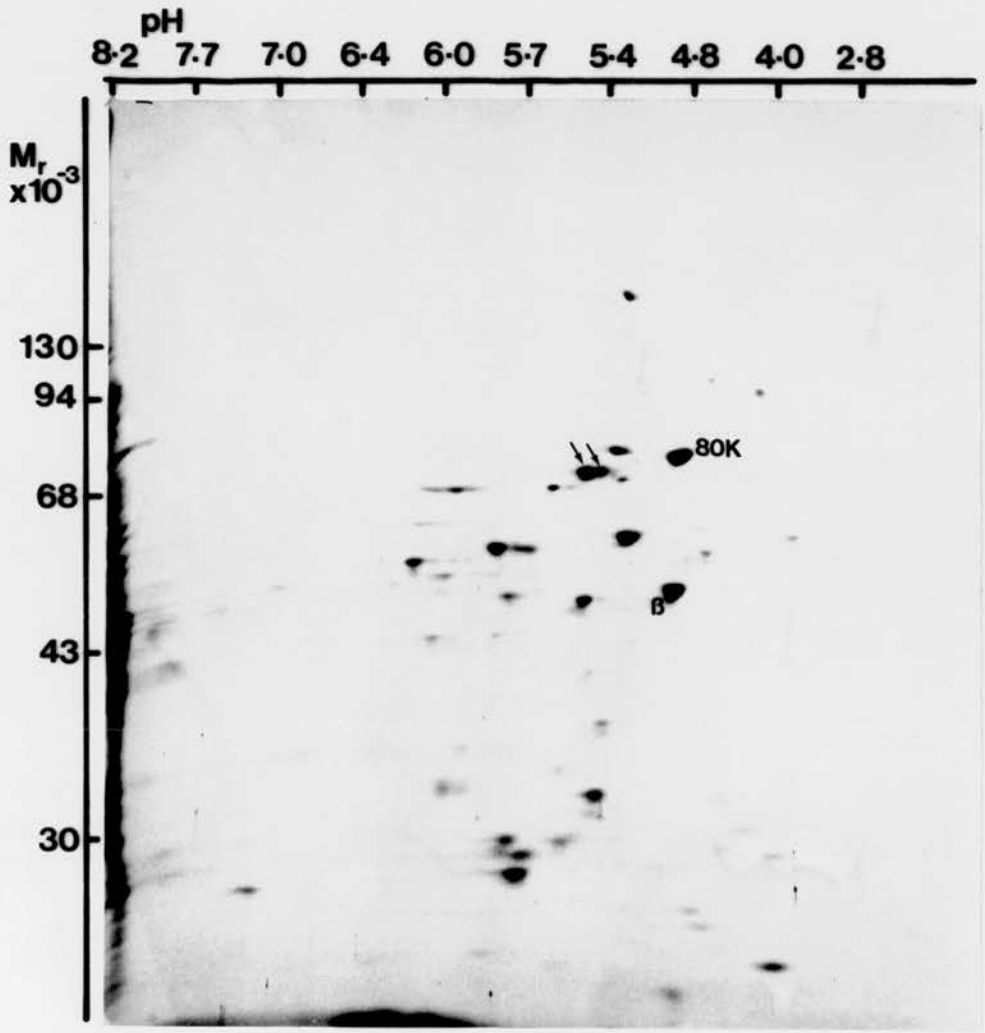
Figure 4.6 shows a two-dimensional electrophoretogram of mitochondrial membrane proteins. One of these ($M_r$80,000; pI5) was detected in all the microsomal fractions, in particular in the RER membranes which equilibrate at a similar buoyant density on sucrose gradients as mitochondrial membrane. These proteins were further resolved by solubilisation and phase separation in Triton X-114. Figure 4.7b shows that the majority of the Coomassie staining polypeptides fractionate in the aqueous phase, in particular the 80K protein and two soluble proteins which migrate in a position identical to the chromaffin granule membrane ATPase I 70K polypeptide. The major polypeptide in the detergent phase (Figure 4.7b) is the catalytic subunit of the $F_1F_0$-ATPase (as identified by decoration with antiserum raised against yeast $F_1$-ATPase).

### 4. Microsomal Fractions.

Figure 4.8 shows electrophoretograms of the microsomal fractions (Figure 3.1, fractions 6,7&8) stained with Coomassie blue. The staining patterns show a great deal of similarity, but they do supplement the one-dimensional electrophoretic analysis, showing that the major soluble protein ($M_r$68,000, pI5.8) of the Golgi membrane enriched fraction is distinct from chromogranin A. Only the Golgi membrane fraction contains a significant amount of chromogranin A. This protein is barely detectable by Coomassie staining in the RER-membranes (Figure 4.8c). Comparatively little chromogranin A was present in the plasma membrane (Fraction 6) and the major Coomassie staining protein was the Golgi fraction protein of $M_r$68,000.

Two relatively major proteins of the Golgi membrane fraction

Figure 4.6. Two-Dimensional Electrophoretogram of Mitochondrial Membrane Proteins.




Mitochondrial membranes (312µg) were solubilised in 10% β-octylglucoside then diluted with 2vol. of focusing sample buffer (Chapter 2). After centrifugation for 60min at 40,000rpm in an SW50.1 rotor samples were loaded at the basic end of the focusing gel. The β-subunit of the $F_1$-ATPase is indicated as is an $M_r$ 80,000, pI5 protein singled out as it is one which appears in many of the membrane fractions analysed.

**Figure 4.7. Two-Dimensional Electrophoretogram of Mitochondrial Membrane Proteins After Solubilisation and Phase Separation in Triton X-114.**

Mitochondrial membranes purified by density gradient centrifugation were solubilised and phase separated in Triton X-114 as described in Chapter 5 where a one dimensional electrophoretogram is shown in Figure 5.11c.

**(a).** shows the detergent phase (300µg of protein) which is dominated by the $\beta$-subunit of the $F_1$-ATPase.

**(b).** shows the aqueous phase (300ug of protein) and a polypeptide ($M_r$ 80,000, pI5) is indicated which may be identical to a protein with identical characteristics in microsomal membrane fractions (see Figures 4.8&4.9). Arrows indicate proteins migrating in positions similar to chromaffin granule membrane ATPase I 70K polypeptides.

Samples were prepared as described in Figure 4.6.

**Figure 4.8. Two-Dimensional Electrophoretogram of Microsomal Membrane Fractions.**

**(a).** Plasma Membranes (Fraction 6; 360µg of protein).

**(b).** Golgi-enriched membranes (Fraction 7; 300µg of protein).

**(c).** RER-enriched membranes (Fraction 8; 300µg of protein.

CgA, chromogranin A; $Cytb_{561}$, cytochrome $b_{561}$. Note that CgA is absent from the RER-enriched membrane fraction.

**Figure 4.9. Two-Dimensional Electrophoretogram of Microsomal Membrane Fractions Solubilised and Phase Separated in Triton X-114.**

This figure shows phase separations of Golgi-enriched membranes (a&b) and RER-enriched membranes (c&d). Both these fractions are dominated by soluble proteins.

Gels, 7-15%, stained with Coomassie blue. Samples (300µg of protein) were lyophilised, then solubilised in [x1] focusing sample buffer. Standard proteins were as shown in Figure 4.1.

**Figure 4.7.**

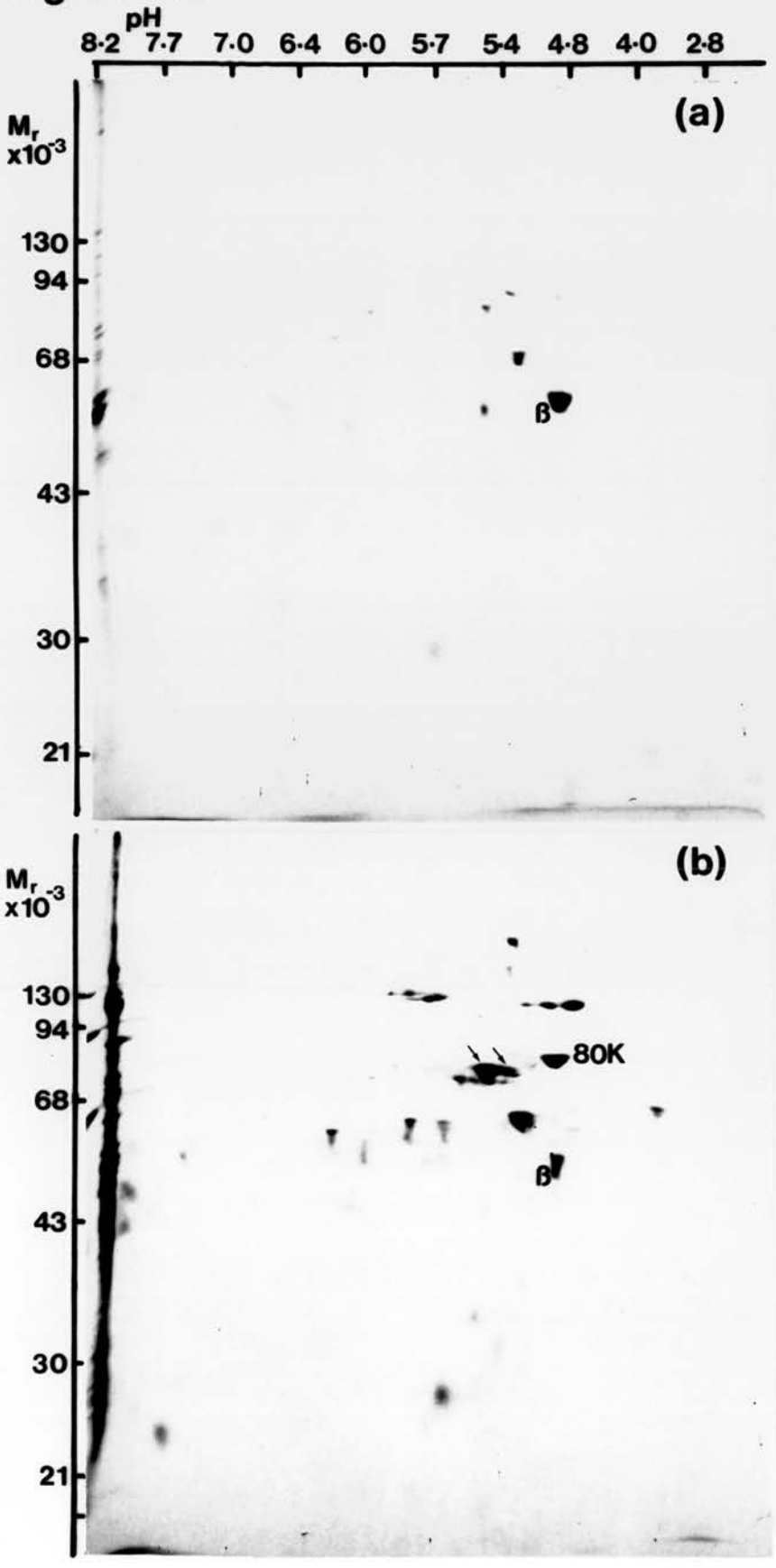

pH
8·2
7·7
7·0
6·4
6·0
5·7
5·4
4·8
4·0
2·8
(a)
$M_r$ x10$^{-3}$
130
94
68
43
30
21
β
(b)
$M_r$ x10$^{-3}$
130
94
68
43
30
21
80K
β

Figure 4.8.

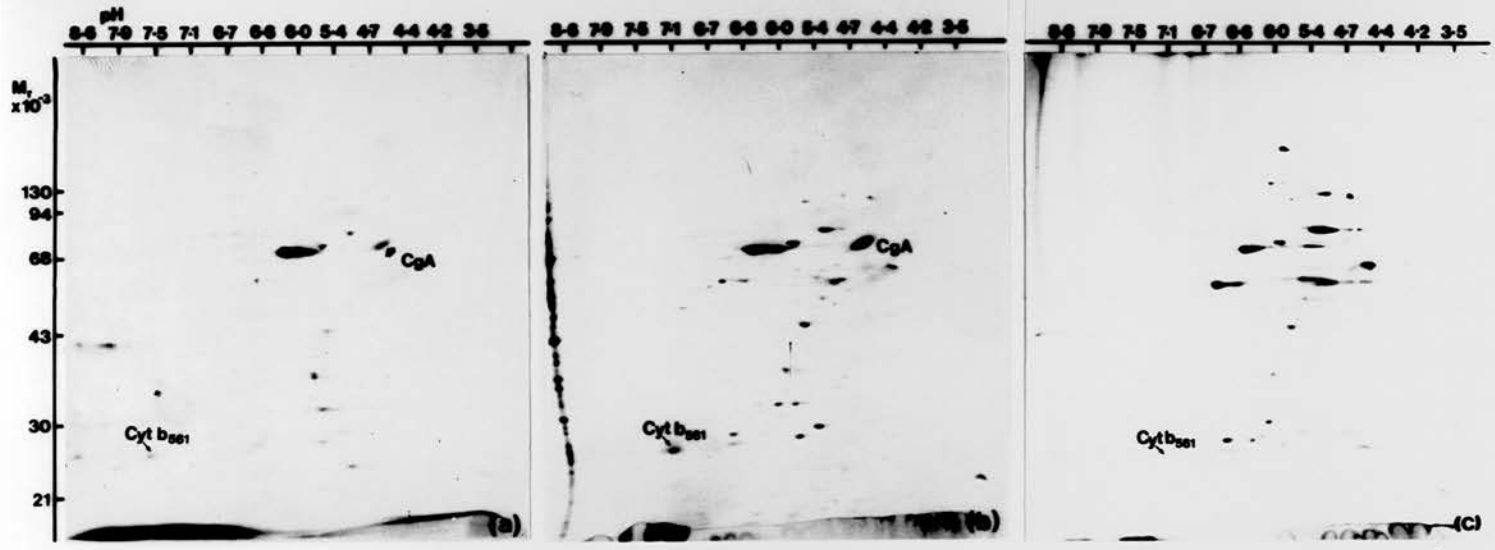
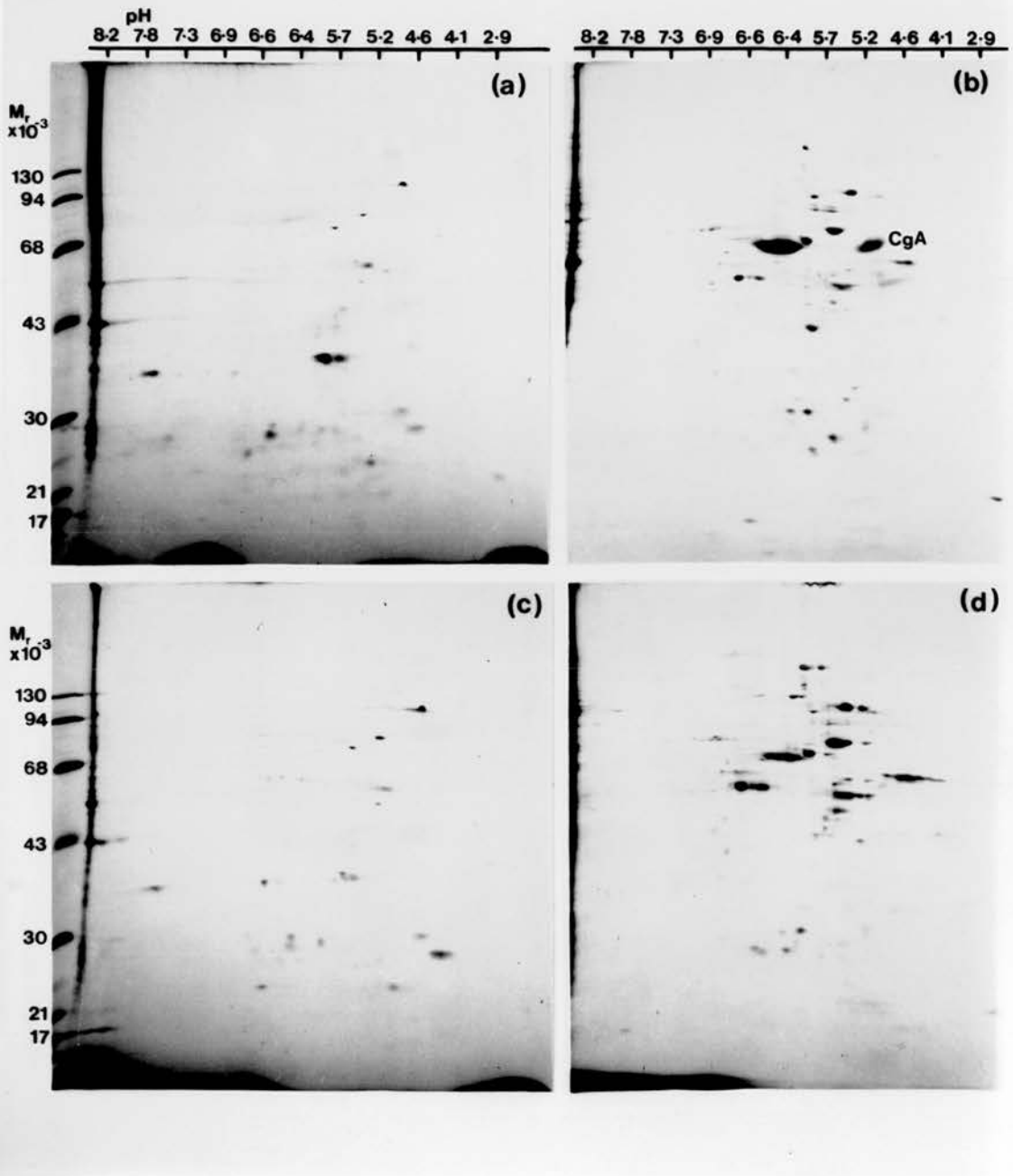

pH
8·6 7·9 7·5 7·1 6·7 6·6 6·0 5·4 4·7 4·4 4·2 3·5
$M_r$ x10$^{-3}$
130
94
68
43
30
21
CgA
Cyt $b_{561}$
(a)
(b)
(c)

**Figure 4.9.**


pH
8·2 7·8 7·3 6·9 6·6 6·4 5·7 5·2 4·6 4·1 2·9
(a)
(b)
(c)
(d)
$M_r$ x10$^{-3}$
130
94
68
43
30
21
17
CgA

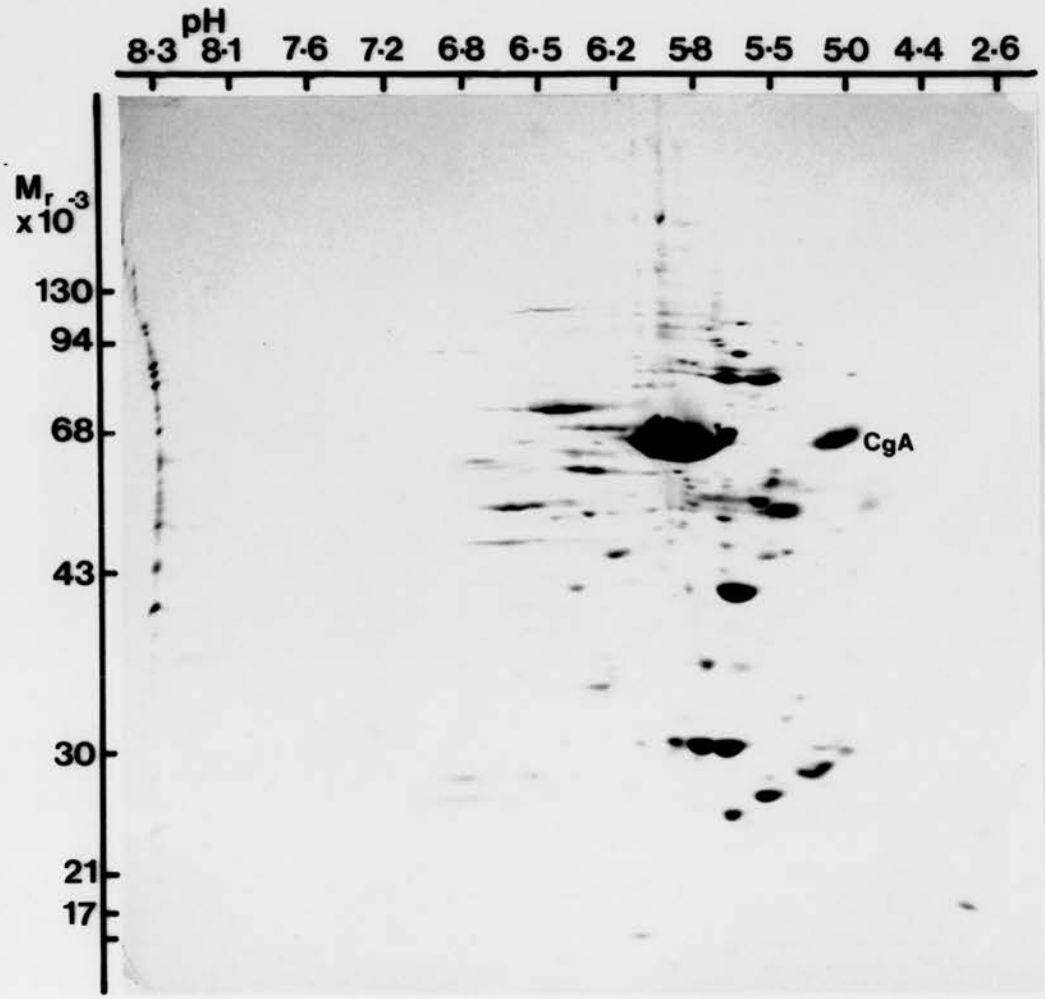
are chromogranin A and cytochrome $b_{561}$. This may indicate contamination by chromaffin granule membranes, although when one compares the ratio of these two proteins in Figure 4.8b with the chromaffin granule membranes shown in Figure 4.5, it is clearly increased; this suggests that there may be a population of secretory vesicles equilibrating with the Golgi membrane marker galactosyltransferase, since both chromogranin A and the cytochrome in this fraction appear to be identical to the proteins shown in Figure 4.5, as has been confirmed immunologically (see below). It is not possible to conclude that these are endogenous Golgi proteins, however they may at the very least represent the population of immature granules containing newly synthesised and fully glycosylated chromogranin A (see Chapter 6) packaged within recycled chromaffin granule membranes. There was however no appreciable DBH activity associated with this fraction (see Chapter 3).

The RER membrane fraction is particularly striking in that Coomassie blue reveals little chromogranin A and cytochrome $b_{561}$. The localisation of many other proteins of the other proteins in these fractions can a best only be a speculative exercise, best resolved immunologically. Figure 4.9 shows a phase separation of Golgi- and RER-enriched membrane fractions in Triton X114. This figure demonstrates that soluble proteins predominate in these fractions. The detergent phase (containing integral membrane proteins) focused poorly (Figure 4.9a&c), the majority of polypeptides remaining at the basic end of the gel. The gels shown in Figure 4.9 are the best of many attempts in which sample treatment conditions were varied to try to improve the resolution of the Coomassie-staining proteins.

5. Cytosolic Proteins.

Figure 4.10 shows a two-dimensional electrophoretogram of the proteins of the post-microsomal supernatant (Fraction 4 of Figure 3.1) which should reflect the cytoplasmic protein content. This gel is dominated by the protein which on previous electrophoretograms has been associated with the Golgi fraction (Figure 4.8). This protein ($M_r$68,000; pI5.8) is clearly the major adrenal medullary cytoplasmic protein and its electrophoretic behaviour is identical to that of bovine serum albumin. While adrenal medullary tissue may be contaminated to some extent by bovine plasma proteins, it is shown in chapter 6 that this protein is synthesised by isolated chromaffin cells, purified on gradients of Percoll. It will also be shown that its presence in the Golgi fraction may be for the post-translational addition of fatty acid. There is a relatively small amounts of chromogranin A in this fraction (compare this electrophoretogram with that of total cell proteins Figure 6.3), where it can be seen that chromogranin A is by far the most abundant adrenal medullary protein. This analysis suggesting that the homogenisation procedure used did not cause significant rupture of organelles. The identity of many of the other cytoplasmic proteins is unknown.

Figure 4.10. Two-Dimensional Electrophoretogram of the Post-Microsomal Supernatant.




The post-microsomal supernatant (Fraction 4 of Figure 3.1) was prepared in an identical manner to that described for chromaffin granule lysate (see Chapter 2). 300µg of protein was focused and then analysed on a 7-15% gel.

**Immunoblotting Analysis.**

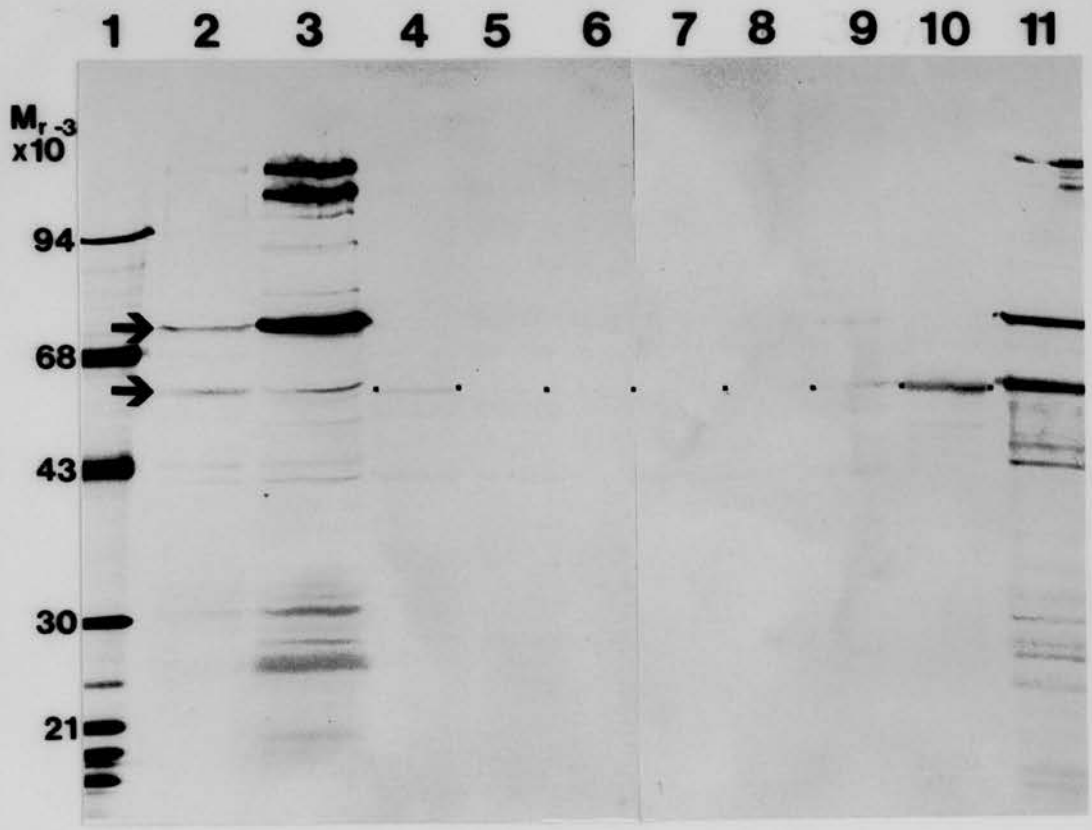
The marker enzyme analysis (Chapter 3) suggested that while the three microsomal subfractions were enriched in, Golgi, RER and plasma membrane, each clearly cross-contaminated the other. In addition the highly purified plasma membrane fraction isolated here in comparitively low abundance, did not sediment at the same density reported by previous workers, whose fractions sedimented at densities identical to the Golgi membrane fraction isolated here. Thus there is the possibility that Band I (Figure 3.2) is a 'light Golgi' fraction similar to the fraction isolated from rat liver (i.e. $G_{F1}$ fraction; Bergeron et al., 1978). The enzyme marker data for RER membranes was not particularly convincing and so immunoblotting and lectin overlay analysis was used to try and resolve the origin of the membrane fractions.

**1. Docking Protein.**

It was difficult to demonstrate unequivocally the presence of RER in microsomal band III, since glucose-6-phosphatase was an unsatisfactory marker, and both glucosidase II and RNA analyes showed rather heterogeneous distributions. To confirm the presence of RER membranes in fraction 8 an integral membrane protein which is localised solely in the RER was required. An antiserum to the $M_r$60,000 fragment of the docking protein of dog pancreas microsomes (gift from David Meyer, EMBL, Heidelberg) fulfilled this criterion and was used to screen adrenal medullary fractions.

Figure 4.11 shows a composite autoradiogram of immunoblots of microsomal fractions probed with antidocking protein antibodies. Track 2 shows dog pancreas microsomes and track 3, the same membranes after a sodium carbonate wash, showing that the $M_r$72,000

Figure 4.11. Immune Blot With Anti-Docking Protein.




Membrane fractions 100µg of protein/track were separated on a 7-15% one-dimensional gel under reducing conditions then transferred to nitrocellulose, probed with antiserum (1:100 dilution) then decorated with [$^{125}$I]-Protein A.
Track 1, standard proteins as described in Figure 4.1 radiolabelled with [$^{14}$C]-formaldehyde; track 2, dog pancreas microsomes; track 3, sodium carbonate washed dog pancreas microsomes; track 4, adrenal medullary RER-enriched membranes; track 5, adrenal medullary Golgi-enriched membranes; track 6, chromaffin granule membranes; track 7, sodium carbonate washed plasma membranes; track 8 sodium carbonate washed Golgi membranes; track 9, proteins washed from RER-membranes; track 10, sodium carbonate washed adrenal medullary RER-membranes; track 11, dog pancreas microsomes (a different preparation to that shown in track 2). The arrows indicate the docking protein ($M_r$72,000) and the membrane fragment ($M_r$60,000), resistant to proteolysis, to which the antibody was raised.

protein recognised by the antiserum is indeed an integral protein. Track 4 contains RER membranes (Band III, Figure 3.2) and shows a comparitively minor band of $M_r$72,000 is recognised by the antiserum. The major polypeptide labelled in this fraction is one of $M_r$60,000 which suggests that much of the adrenal medullary docking protein has undergone proteolysis during isolation of the membranes. This is also demonstrated for a dog pancreas preparation in track 7. Track 8 shows RER-membranes after washing with sodium carbonate in which the majority of the docking protein is present as the $M_r$60,000 species. There was no significant amount of this polypeptide in either Golgi membranes (track 5&10) or plasma membrane (track 11). Chromaffin granule membranes (track 6) were not labelled with the antiserum.

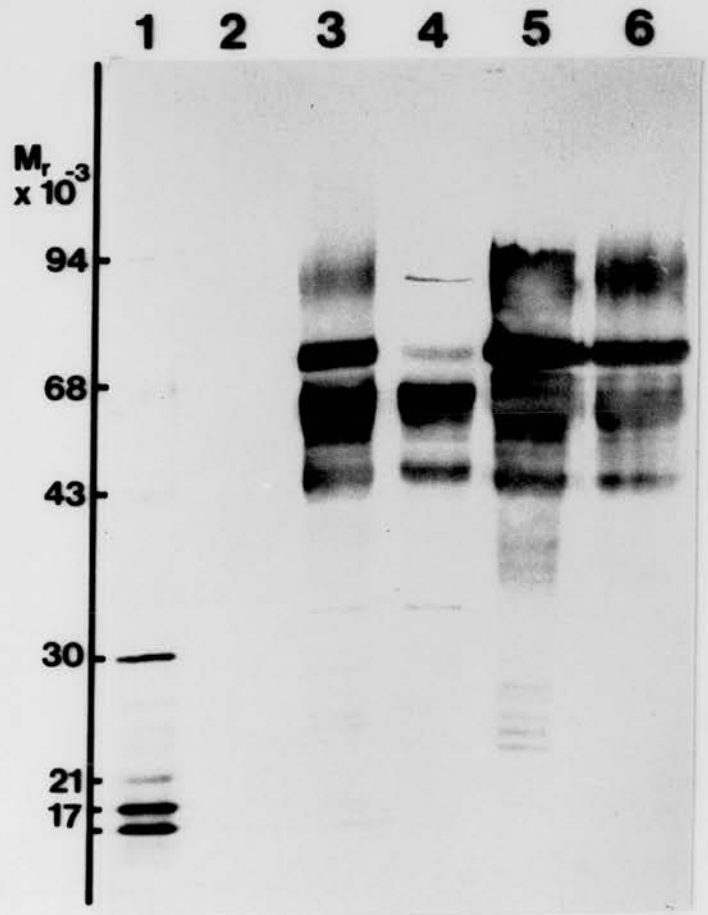
## 2. Protein Biogenesis.

### a. Chromogranin A.

The biogenesis of chromogranin A has been readily studied by following cellular synthesis with [$^{35}$S]-methionine (Chapter 6). However, no membrane proteins were identified on fluorograms of [$^{35}$S]-methionine labelled cell lysates, and while radiolabelled-DBH was immunoprecipitated from these it appeared to be only the soluble form of the enzyme. Therefore, to identify precursors to the integral membrane proteins DBH and cytochrome $b_{561}$, subcellular fractions enriched in RER and Golgi membrane markers were analysed by immunoblotting of one- and two-dimensional electrophoretograms. Since unglycosylated precursors of chromogranin A were readily identified in [$^{35}$S]-methionine labelled cell lysates this secretory protein was used as a control for the validity of this approach.

Figure 4.12 shows a one-dimensional immunoblot with anti-chromogranin A antiserum of the soluble proteins washed from each of the microsomal fractions. Band I is striking in that no chromogranin A appears to be associated with this fraction. This is in contrast to Coomassie staining which appears to reveal some chromogranin A (Figure 4.8) and may reflect preparative variations. The mature chromaffin granule polypeptide pattern of chromogranin A shown in Figure 12, Track 5 reveals many low $M_r$ proteolysis products. This antiserum also recognises a highly glycosylated form of chromogranin A. The chromogranin A polypeptides associated with the chromaffin granule membrane are shown in Track 6. Only the high $M_r$ glycosylated species appear to be associated with the membrane. The chromogranin A species associated with the Golgi membrane fraction are almost identical to the granule lysate polypeptide pattern. However, there do appear to be minor differences in the proteolytic fragments associated with this fraction. The RER fraction is strikingly different to the other microsomal fractions. There is very little of the mature form of chromogranin A identified by this very sensitive procedure. However, as with the other fractions chromogranin A appears to have undergone extensive proteolysis. The molecular weights of the breakdown products appear to be slightly larger than those of the Golgi membrane fraction and the granule lysate fraction. This suggests that these proteolysis products may arise from some form of the polypeptide other than the mature species, such as the unglycosylated form of the protein; alternatively hydrolysis may be catalysed by a different proteolytic enzyme in this fraction. This proteolytic activity does not appear to have been affected by the PMSF and benzamidine included in the preparation buffers. The highly glycosylated (proteoglycan) form of

Figure 4.12. One-dimensional Immune Blot of Soluble Proteins of the Microsomal Fractions Labelled With Anti-Chromogranin A.




Microsomal fractions were washed with sodium carbonate as described in Chapter 2. The proteins were separated under reducing conditions on a 7-15% gel then blotted and probed with a 1:100 dilution of anti-chromogranin A antiserum.

Track 1, standard proteins as described in Figure 4.1; track 2, soluble proteins associated with the plasma membrane fraction (20µg); track 3, soluble proteins associated with Golgi membranes (20µg); track 4, soluble proteins associated with the RER membranes (20µg); track 5, chromaffin granule lysate proteins (10µg); track 6, chromaffin granule membrane proteins (20µg).

chromogranin A (Rosa et al., 1985a) is not present in Fraction 8, again suggesting that this is a pre-Golgi compartment. The nature of the polypeptide of $M_r$90,000 is unknown. It is possible that it may be a precursor form of the 'proteoglycan', but the identity of this polypeptide and its relationship to chromogranin A is controversial.

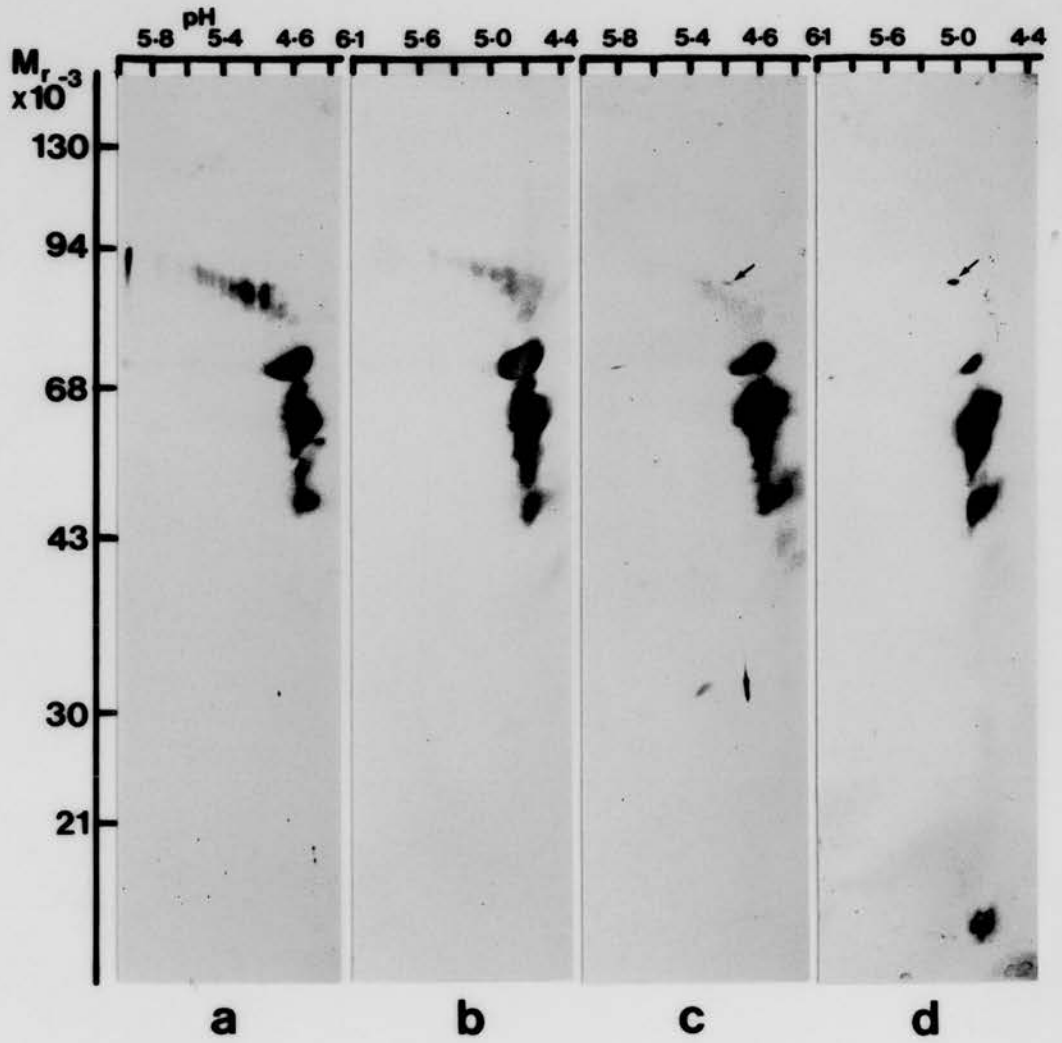
Two dimensional analysis of the proteins released by $Na_2CO_3$ wash from these fractions confirms these observations. Figure 4.13 shows immune replicas of gels decorated with anti-chromogranin A antiserum. Residual chromogranin A that is associated with granule membranes is essentially indistinguishable from that released from granules by lysis (Figure 4.13a&b), and material isolated from the Golgi-enriched fraction is essentially similar. This applies to the degradation products as well as the major species, so one cannot rule out the possibility of these arising from a small contamination of granule membranes, obliterationg any precursor forms.

The RER-enriched fraction contains virtually no mature chromogranin A: the $M_r$70,000 species resembles an early precursor, before glycosylation (see Chapter 6). The high $M_r$ proteoglycan is completely absent. The low $M_r$ species are abundant, but not all the species seen in the other fractions seem to be present. This suggests that the chromogranin A species accumulate, owing perhaps to removal of some targeting information. This is highly speculative however.

b. Dopamine $\beta$-hydroxylase.

Figure 4.14a shows a representative two-dimensional autoradiogram of the labelling pattern obtained with microsomal membrane fractions and chromaffin granule membrane or lysate proteins decorated with anti-DBH antiserum. Both this analysis and

Figure 4.13. Two-Dimensional Immune Blot of Microsomal Fraction Soluble Proteins With Anti-Chromogranin A.




Immune blots of the acid region of two-dimensional gels of the fractions shown in Figure 4.12, aligned using radioactive molecular weight markers run on the same gel.

(a). Chromaffin granule membranes (25µg of protein).
(b). Chromaffin granule lysate (10µg of protein).
(c). Golgi-content proteins (40µg of protein).
(d). RER-content proteins (40µg of protein).

Anti-chromogranin A antiserum was used at a dilution of 1:100. The arrow indicates a polypeptide (not found in chromaffin granule membrane fractions) whose identity is unknown.

Figure 4.14. One- and Two-Dimensional Immune Blots With Anti-Dopamine $\beta$-hydroxylase.

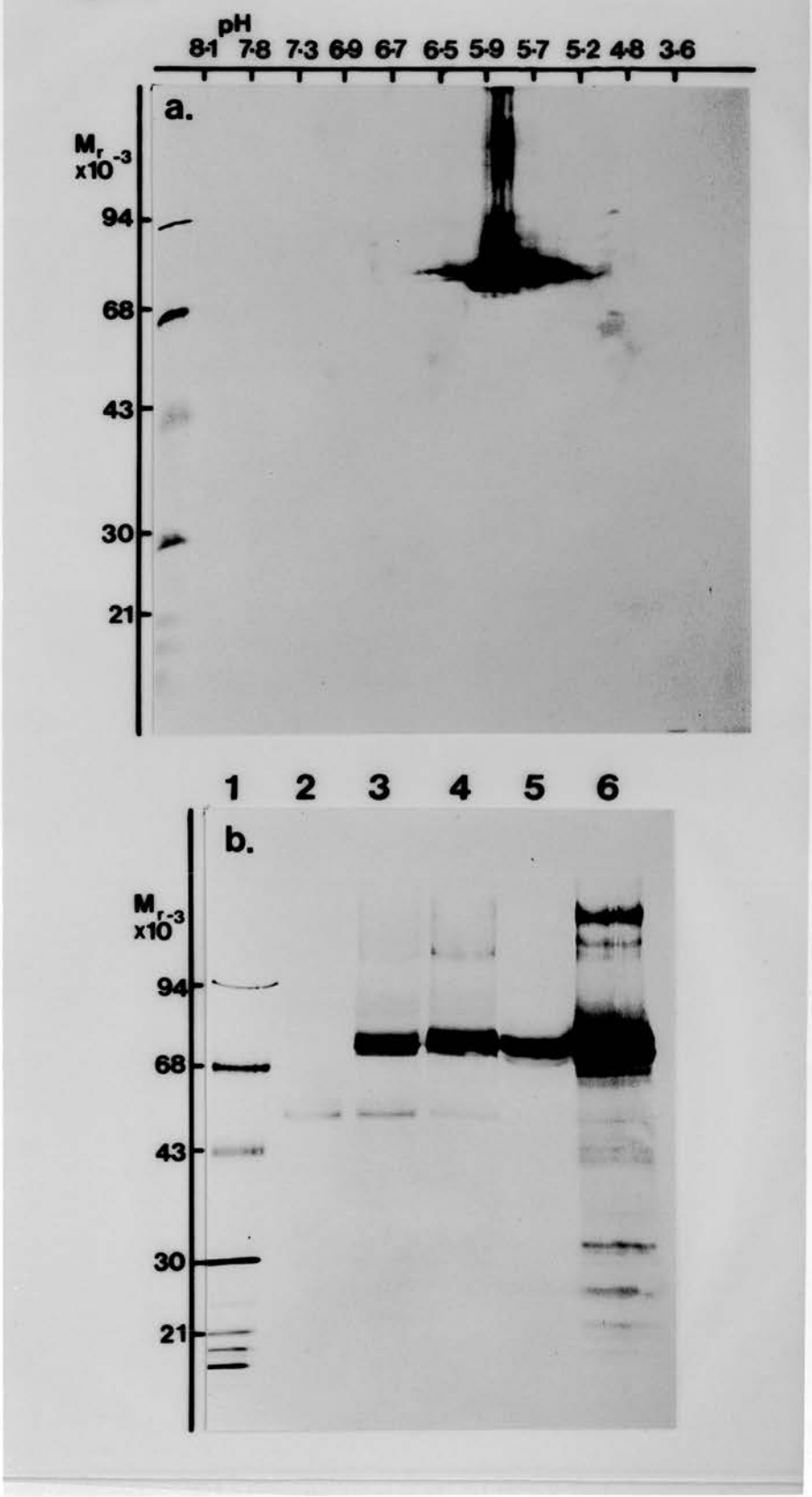
(a). Autoradiogram of a two-dimensional gel of chromaffin granule membranes (50µg) blotted and probed with antiserum to DBH diluted 1:100 and decorated with [$^{125}$I]protein A.

(b). Autoradiogram of a 7-15% one-dimensional gel of proteins washed from microsomal fractions by sodium carbonate blotted and probed with antiserum to DBH diluted 1:100. Track 1, radiolabelled standard proteins; track 2, solubilised plasma membrane proteins (50µg); track 3, solubilised RER-membrane proteins (100µg); track 4, solubilised Golgi-membrane proteins (100µg); track 5, chromaffin granule lysate proteins (50µg); track6, chromaffin granule membrane proteins (100µg).
Note: while the two-dimensional pattern of DBH showed extensive heterogeneity when probed with antisera, the resolution was not improved at lower protein loadings nor with shorter exposure times.

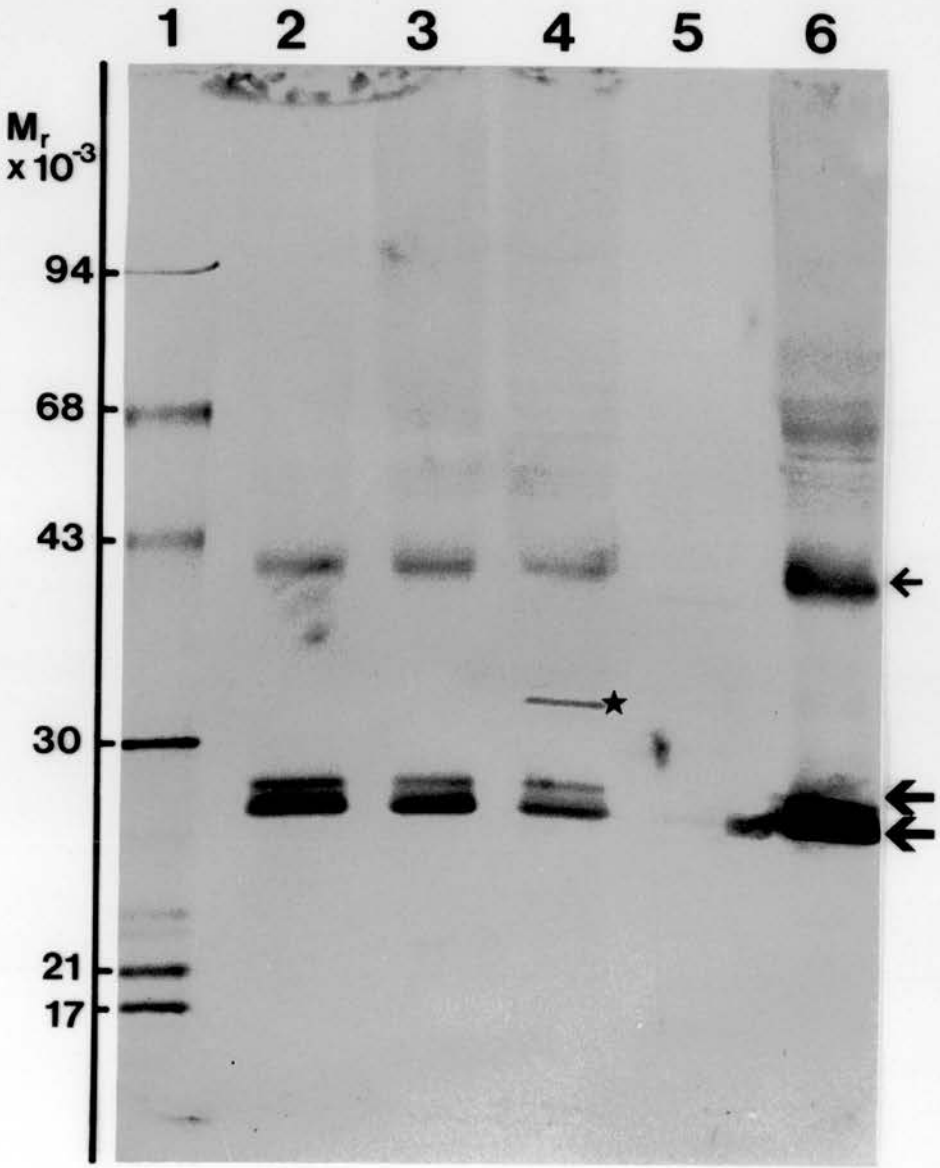
Figure 4.15. One-Dimensional Immune Blot of Microsomal Membrane Fractions With Anti- Cytochrome $b_{561}$.

An 8-15% gel run under reducing conditions, each track containing 50µg of protein. Track 1, radiolabelled standard proteins (see Figure 4.1); track 2, plasma membrane; track 3, Golgi membranes; track 4, RER membranes; track 5, mitochondrial membranes; track 6, chromaffin granule membranes. The bold arrows indicate the characteristic cytochrome doublet while the smaller arrow shows the position of an aggregate form of the cytochrome. The additional band indicated in track 4 is present when preimmune serum replaces the antiserum.

Figure 4.14.


pH
8·1 7·8 7·3 6·9 6·7 6·5 5·9 5·7 5·2 4·8 3·6
a.
$M_r$ x10$^{-3}$
94
68
43
30
21
1 2 3 4 5 6
b.
$M_r$ x10$^{-3}$
94
68
43
30
21

# Figure 4.15



one-dimensional analysis failed to provide any reliable indication of differences between microsomal and chromaffin granule membrane fractions. There were minor differences in the proteolytic degradation products in the different microsomal fractions. DBH is cotranslationally N-glycosylated in the RER, and any differences in the glycosylation states at this early stage of processing may be too subtle to be reliably identified by this technique.

c. Cytochrome $b_{561}$.

Figure 4.15a shows a one-dimensional immune blot of the microsomal fractions decorated with anticytochrome $b_{561}$ antiserum. Track 6, which contains chromaffin granule membranes, shows the characteristic labelling of the cytochrome doublet Apps et al., 1980a). The cross-reacting polypeptide ($M_r$ 40,000) is an aggregate of the cytochrome (discussed by Apps et al., 1984).

In contrast to analysis with the antisera to chromogranin A and DBH the plasma membrane-enriched fraction (Band I) contains cytochrome $b_{561}$, as do the other microsomal fractions. There were no candidates for precursor proteins; the additional polypeptide decorated in the RER fraction (track 4) was also identified on an immune blot using preimmune serum. Two-dimensional analysis showed no differences in the isoelectric focusing pattern of the cytochrome from the three microsomal fractions. Purified mitochondrial membrane fractions did not contain cytochrome $b_{561}$ (track 5). The absence of DBH and chromogranin A suggested that microsomal band I (Fraction 6) was not contaminated by chromaffin granule membranes. It is therefore difficult to explain the presence of the cytochrome in this fraction simply on the basis of contamination. Its presence in each microsomal fraction, as shown by immune blotting,

confirms the spectrophotometric analysis presented in Chapter 3, which however, indicated that there was apparently no enrichment of the cytochrome in the microsomal fractions. This latter analysis also showed that the cytochrome present contains its complement of haem in each case. It would obviously be interesting to investigate whether there is indeed an additional plasma membrane localisation of cytochrome $b_{561}$ in this tissue.

An initial aim of this work was to identify biogenic precursors to major integral proteins of the granule membrane using the sensitive immune blotting technique. However, this approach depended for its success on (i) there being adequate amounts of precursors accumulated in non-granule compartments for identification and (ii) on antisera raised to the mature granule polypeptides recognising the denatured precursors on blots. In addition, precursors obviously cannot be recognised unless they migrate in a different position on a gel from the mature species. In the case of cytochrome $b_{561}$ (unglycosylated) and DBH (N-glycosylated) it appears that any such differences may not be sufficiently marked for detection. In the case of chromogranin A (O-glycosylated) the putative unglycosylated precursor appeared to be present in the RER-enriched fraction.

**Lectin Overlay Analysis.**

Chromaffin granule membrane preparations contain a number of glycoproteins whose carbohydrate chains are terminated with sialic acid residues. These anionic sugar moieties together with modifications such as phosphorylation and sulphation, which take place in the Golgi complex, give these proteins characteristic focusing patterns on two-dimensional gels. These can be decorated with radioiodinated lectins (Gavine et al., 1984). In the absence of antibodies to these glycoproteins I attempted to identify precursor polypeptides to the major membrane glycoproteins using lectins (shown in Table 4.1) to screen one and two-dimensional replicas of the adrenal medullary microsomal fractions. Glycoproteins in the RER containing only N-linked high-mannose core oligosaccharides will of course not contain complex sugars and should therefore show little hetergeneity during focusing.

**1. Lectin Screening.**

Table 4.1 shows the lectins used to screen the microsomal fractions for glycoproteins. Ricin 1 agglutinin and soybean agglutinin showed no significant labelling of adrenal medullary proteins. Horseshoe crab lectin, supposedly specific for sialic acid residues, showed no significant labelling of chromaffin granule membrane proteins, but did label many of the microsomal proteins without specificity. Many proteins in the mitochondrial membrane fraction were also labelled by this lectin. Labelling with horse gram lectin and Ricin 2 agglutinin, was similar to horseshoe crab lectin. These preliminary studies suggested that lentil lectin, concanavalin A and to some extent wheat germ agglutinin would be useful for screening analysis.

Table 4.1. Source and Specificities of the Lectins Used for Overlay Analysis.

| Lectin. | | Source. | Specificity. |
|---|---|---|---|
| Concanavalin A. | (Con A) | Jack Bean *Canavalia ensiformis.* | D-Man D-Glc D-GlcNAc. |
| Horse Gram. | (DBA) | *Dolichos bifloris.* | D-GalNAc |
| Horse Shoe Crab Agglutinin. | (HSC) | *Limulus polyphemus.* | Sialic Acid. |
| Lentil Lectin. | (LCL) | *Lens culinaris.* | D-Man D-Glc D-GlcNAc |
| Peanut Agglutinin. | (PNA) | *Arachis hypogaea.* | D-Gal |
| Ricins 1&2. | (RCA-1&2) | Castor Bean. *Ricinis communis.* | D-Gal D-Gal D-GalNAc. |
| Soybean Agglutinin. | (SBA) | *Ulex europaeius.* | L-Fucose |
| Wheat Germ Agglutin. | (WGA) | *Triticum vulgaris.* | D-GlcNAc. |

Man, mannose; Glc, glucose; GlsNAc, N-acetylglucosamine; Gal, galactose; GalNAc, N-acetylgalactosamine.

2. Concanavalin A.

Figure 4.16 shows a one-dimensional concanavalin A overlay of adrenal medullary membrane fractions; an identical gel labelled in the presence of $\alpha$-methylmannoside showed no labelling with concanavalin A. The Golgi-enriched microsomal membranes contain significant amounts of a high $M_r$ (200,000) concanavalin A binding protein, present in only small amounts in chromaffin granule membranes (see Table 2 of Gavine et al., 1984). The RER membranes are also specifically enriched in another high $M_r$ (150,000) glycoprotein. The concanavalin A binding glycoproteins present in the mitochondrial membrane fraction (Figure 4.16a, track 2) appear to reflect their contamination by microsomal glycoproteins.

3. Lentil Lectin.

Figure 4.17 shows an autoradiogram of a one-dimensional overlay of sodium carbonate washed microsomal membranes decorated with [$^{125}$I]-lentil lectin. None of the proteins in the mitochondrial fraction bind this lectin. Plasma membrane (Fraction 6) in track 3 however contains a major lentil lectin binding protein of $M_r$ 32,000. There is relatively little contamination of the Golgi-enriched membrane fraction with this major component of Band I (Figure3.1, fraction 6). No significant amount of this protein is detectable in the other fractions. Two-dimensional overlays with lentil lectin (not shown) of plasma membranes show that the $M_r$32,000 protein is basic (pI8). Lentil lectin also decorates another glycoprotein $M_r$35,000 which is characteristically enriched in the RER fraction. There is a small amount of this protein in the Golgi-enriched membrane fraction, indicative of a slight cross-contamination between these fractions. Chromaffin granule membranes are characterised by glycoprotein III

Figure 4.16. [$^{125}$I]Concanavalin A Overlay of Microsomal Fractions.

A comparison of the different membrane fractions isolated from the adrenal medulla on a 7-15% one-dimensional gel under reducing conditions. Each track was loaded with 50µg of protein: track 1, radiolabelled standard proteins (see Figure 4.1); track 2, mitochondrial membranes; track 3, RER membranes (Fraction 8); track 4, Golgi membranes (Fraction 7); track 5, chromaffin granule membranes.

The separated proteins were decorated with radioiodinated lectins as described in Chapter 2 (page 67). Mitochondrial membranes (Figure 3.1 fraction 10) were further purified on a continuous gradient of sucrose (Figure 3.11, page 126). An identical Coomassie stained gel of these fractions is shown in Figure 4.1 (tracks 1-5).

Figure 4.17. [$^{125}$I]-Lentil Lectin Overlay of Microsomal Fractions.

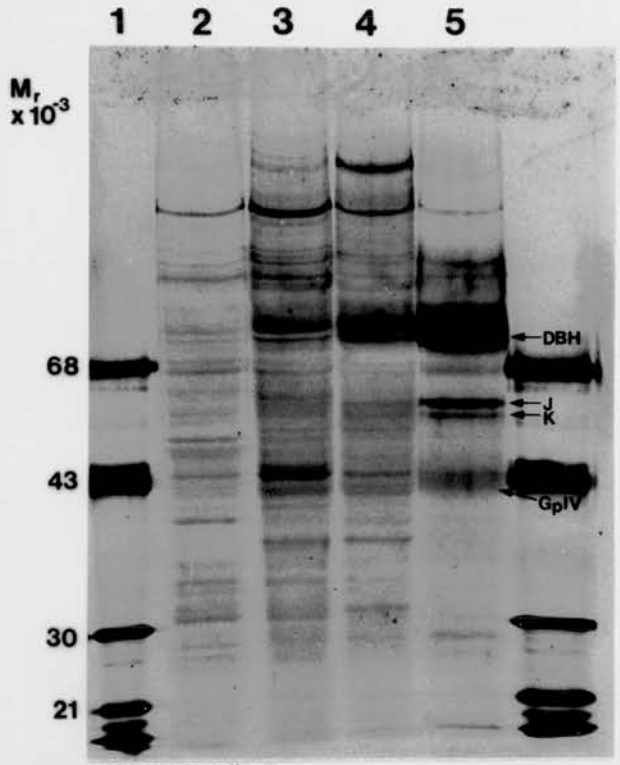
This figure shows an autoradiogram of the lentil binding proteins in membrane fractions tht have been washed with sodium carbonate. Each track contains 100µg of protein: track 1, radiolabelled standard proteins (as described in Figure 4.1); track 2, mitochondrial membranes; track 3, plasma membranes (Fraction 6); track 4, Golgi membranes (Fraction 7); track 5, RER membranes (Fraction 8); track 6, chromaffin granule membranes.

Figure 4.18. Two-Dimensional [$^{125}$I]-Concanavalin A Overlays of Microsomal Membrane Fractions.

Autoradiograph of microsomal membrane fractions which have been washed with sodium carbonate then washed by dilution in 10mM-Hepes NaOH, pH7 and centrifugation. Membrane pellets were lyophilised then solubilised in [x1] focusing sample buffer, clarified by high speed centifugation then 300µg of protein loaded for focusing.

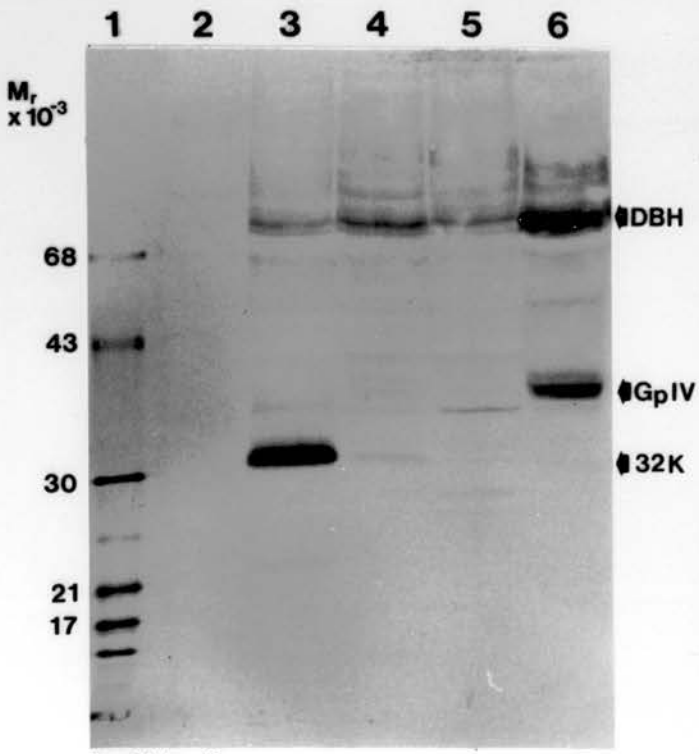
Chromaffin granule membranes are shown in Figure 5.8a and should be compared with the following: (a) RER Membranes (Figure 3.1 Fraction 8), (b) Golgi Membranes (Figure 3.1 Fraction 7), (c) Plasma Membranes (Figure 3.1 Fraction 6). Labelled spots: dopamine $\beta$-hydroxylase, DBH; glycoproteins H,J and K,H,J&K; glycoproteins IIa&b, III and IV, GpIIa&b, GpIII, GpIV.

Figure 4.16.



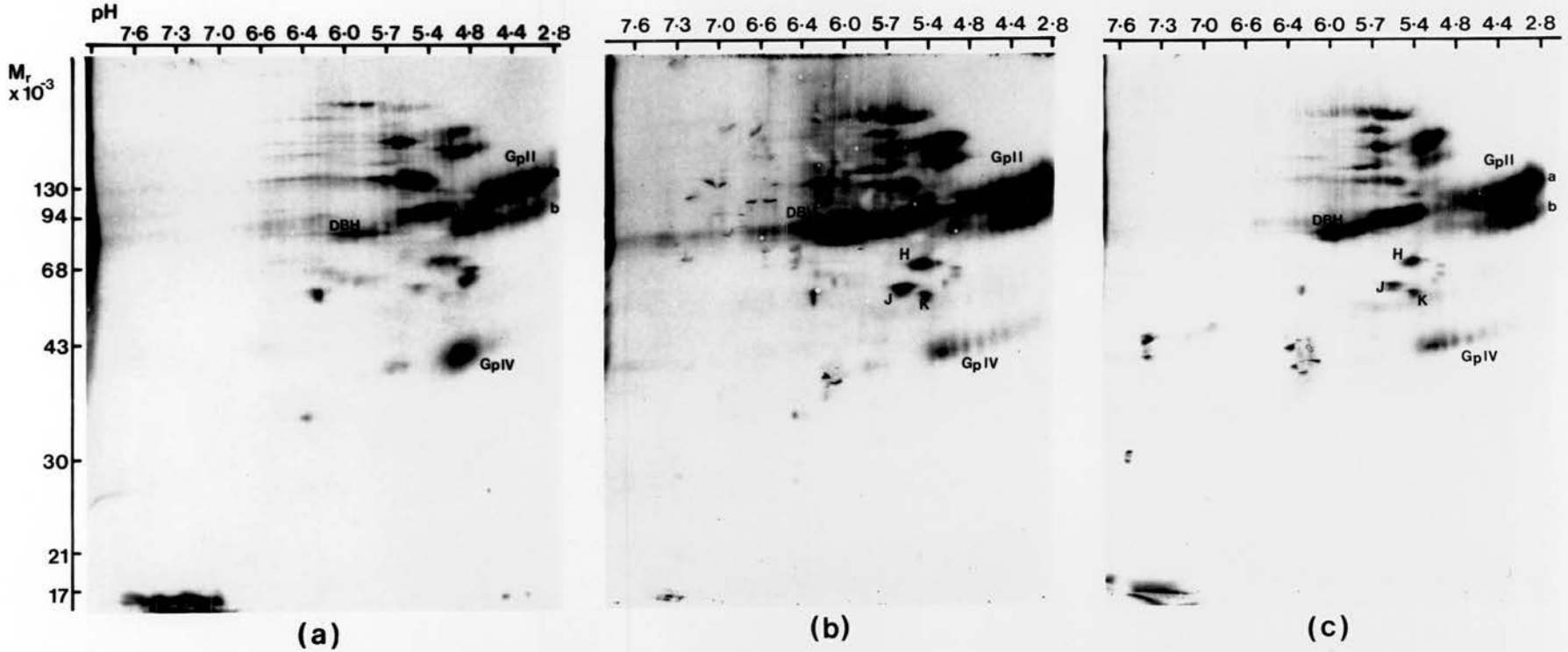

concanavalin A

Figure 4.17.




lentil lectin

**Figure 4.18**


pH
7·6 7·3 7·0 6·6 6·4 6·0 5·7 5·4 4·8 4·4 2·8
$M_r$ x $10^{-3}$
130
94
68
43
30
21
17
GpII
b
DBH
GpIV
H
J
K
a
(a)
(b)
(c)

($M_r$ 37,000), which is the major lentil-binding protein of this fraction (see Gavine et al., 1984 for two-dimensional analysis). This protein cannot be identified in microsomal fractions. Glycoprotein III was also the only glycoprotein to show any significant labelling with wheat germ agglutinin and showed no binding to any proteins in the microsomal fractions. These data suggest that the microsomal membranes were relatively free of contamination by chromaffin granule membranes. However lentil lectin also binds to DBH and this glycoprotein was present to varying degrees in all fractions.

**Two-dimensional Lectin Overlays of Microsomal Fractions.**

Chromaffin granule glycoproteins which show characteristic charge heterogeneity on two-dimensional gels when labelled with [$^{125}$I]-concanavalin are shown in Figure 5.8a. Glycoproteins IIa&b and glycoprotein IV in particular are heavily labelled (see Chapter 5 for Gp nomenclature). However, both these proteins have yet to be unequivocally localised to the chromaffin granule membrane. Only glycoprotein III, which labels specifically with WGA (see Gavine et al., 1984) has been identified as a chromaffin granule membrane protein (Fischer-Colbrie et al., 1982). This glycoprotein was not identified in microsomal fractions by two-dimensional analysis and labelling with $^{125}$I-WGA - presumably the fully glycosylated WGA-binding form is in too low abundance for detection.

Decoration of gels of the plasma membrane enriched fraction with concanavalin A (Figure 4.18c) reveals the presence of some of the glycoproteins familiar from studies of the granule membrane. However, only a relatively small amount of the most abundant chromaffin granule membrane concanavalin A-binding protein, DBH, is

present, as are glycoproteins H,J and K (compare this gel with that of chromaffin granule membranes in Figure 5.8a). This suggests that contamination by granule membranes is relatively minor as was previously suggested by the immune blot analysis of this fraction. In contrast, glycoprotein II (GpII), GpIV and many components with $M_r$ greater than 70,000 and with isoelectric points between 5 and 6 are abundant, and many are presumably genuine plasma membrane components. The major lentil lectin binding protein of this fraction ($M_r$32,000) does not bind concanavalin A

The Golgi membrane enriched fraction (Figure 4.18b) contains a large number of concanavalin A-binding proteins and it resembles a compound gel of both granule membranes (with relatively abundant DBH, H,J and K) and plasma membrane, consistent with the role of the Golgi in the assembly of both organelles. Both GpII and GpIV appear to be concentrated in this fraction (compare Figure 4.18b with Figure 5.8a).

The RER-enriched fraction appears simpler (Figure 4.18a), many of the Golgi membrane spots being reduced or absent. DBH is present only as a single species showing no heterogeneity in either molecular mass or isoelectric point. This is true for the majority of the glycoproteins in this fraction and is consistent with the fact that they are RER glycoproteins, since further addition of complex anionic sugars is a modification restricted to the Golgi complex. Of special note in this fraction is that high-mannose components appear to predominate at the basic ends of both GpIV and GpIIb: obviously these could be unsialylated precursors, however this cannot be proved until we have antibodies to the mature forms of these proteins.

Identification of the SV2 Monoclonal Antigen in Chromaffin Granule Membranes.

Although we have identified many of the major components of the chromaffin granule membrane by electrophoretic and immunological analysis, relatively few of the membrane proteins have been assigned functions. In an attempt to identify those polypeptides which may play important roles in exocytosis and membrane recycling (endocytosis) a number of laboratories have raised monoclonal antibodies to integral membrane glycoproteins, apparently common to neuro-endocrine secretory vesicles, but absent from exocrine secretory vesicles. Three of these monoclonal antibodies have been used in our laboratory to identify their antigens in chromaffin granule membranes: SV2 a trans-membrane glycoprotein $M_r$105-110,000, a component of electroplax vesicles (Buckley and Kelly, 1985); p65, an integral membrane protein isolated from rat brain synaptic vesicles (Matthew et al., 1981) and an integral membrane glycoprotein from brain coated vesicles $M_r$38,000 (Wiedenmann and Franke, 1985).

Characterisation of the SV2 monoclonal was felt to be particularly important since the [$^{125}$I]-concanavalin A overlay studies with adrenal medullary subcellular fractions had indicated a possible candidate for a precursor to glycoprotein IIb (Figure 4.18a) in RER fractions which was apparently devoid of terminal sugars. The electrophoretic characteristics of the SV2 antigen appeared to be similar to glycoprotein II by one-dimensional gel analysis and had SV-2 been identified in chromaffin granule preparations by Buckley and Kelly (1985). Was the SV2 antigen therefore glycoprotein II?

Does Glycoprotein II Contain the Epitope Recognised by the SV2 Monoclonal Antibody?

Figure 4.19 shows in (a) a one-dimensional immune blot and (b) an [$^{125}$I]-lentil lectin overlay of chromaffin granule membranes and lysate run under both reducing and non-reducing conditions (see Figure 4.19 for details). This figure demonstrates that the SV2-antigen is present with identical molecular mass in both reduced and non-reduced membranes (tracks 4&5); it is not detectable among the lysate proteins (tracks 1&2). The SV2 antigen unlike DBH, which was identified by its binding of lentil lectin (Figure 4.19b) was not susceptable to reduction by dithiothreitol; this figure also demonstrates that DBH does not contain the SV2 epitope. By probing the immunoblot with [$^{125}$I]-lentil lectin the position of glycoprotein II was also revealed and by overlaying the immune blot with the autoradiogram of the lentil lectin binding proteins it was shown that the relative molecular mass of the SV2 antigen was in fact distinct from that of glycoprotein II ($M_r$ 75-90,000). Chromaffin granule SV2-antigen has an $M_r$ of between 90,000 and 120,000.

Two-dimensional analysis of the SV2 was problematical. Figure 4.20,a&b shows two-dimensional electrophoretograms with chromaffin granule membrane proteins loaded from the basic and acidic ends respectively. Much of the SV2 antigen precipitates on the top of the focusing gel, protein which does enter the focusing gel precipites at the interface of the second dimension separating gel. A small amount of the antigen appears to have entered from the acidic end. Again, by overlaying with an [$^{125}$I]-lentil lectin autoradiogram of the same gel, the distinct "herring-bone" pattern of glycoprotein II was identified as having focused and entered the

**Figure 4.19. The Chromaffin Granule Membrane SV2-Antigen is Distinct Form Glycoprotein II.**

Chromaffin granule lysate and membrane proteins were analysed under both reducing and non-reducing conditions. Half the sample was reduced with 1mM DTT and then incubated with 5mM Iodoacetamide while the other half was treated only with iodoacetamide. Following electrophoresis and blotting the proteins were probed with SV2-monoclonal antibody (1:100 dilution as described in Chapter 2 page 67) and decorated with SAM-HRP with chloronaphthol as substrate. The same immune blot was then processed for lectin overlay analysis and decorated with [$^{125}$I]-lentil lectin.

**(a).** Immune blot with SV2-monoclonal antibody. Track 1, radiolabelled standard proteins; track 2, non-reduced sDBH (lysate proteins 100µg); track 3, reduced sDBH (lysate proteins 100µg); track 4, partially reduced mDBH (chromaffin granule membranes 100µg); track 5, non-reduced mDBH (chromaffin granule membranes 100µg).

**(b).** $^{125}$I-Lentil lectin overlay of the SV2-monoclonal antibody blot.

**Note:** blocking with BSA in addition to Tween 20 was necessary to obtain low background binding of radioiodinated lectin.

**Figure 4.20. Two-Dimensional Analysis of the Chromaffin Granule Membrane SV2-Antigen.**

Chromaffin granule membranes (300µg of protein) focused from

**(a).** the basic end of a focusing gel.

**(b).** the acidic end. Then probed for the SV2 antigen which precipitates on the top of the separating gel when loaded from the basic end.

**Figure 4.19.**

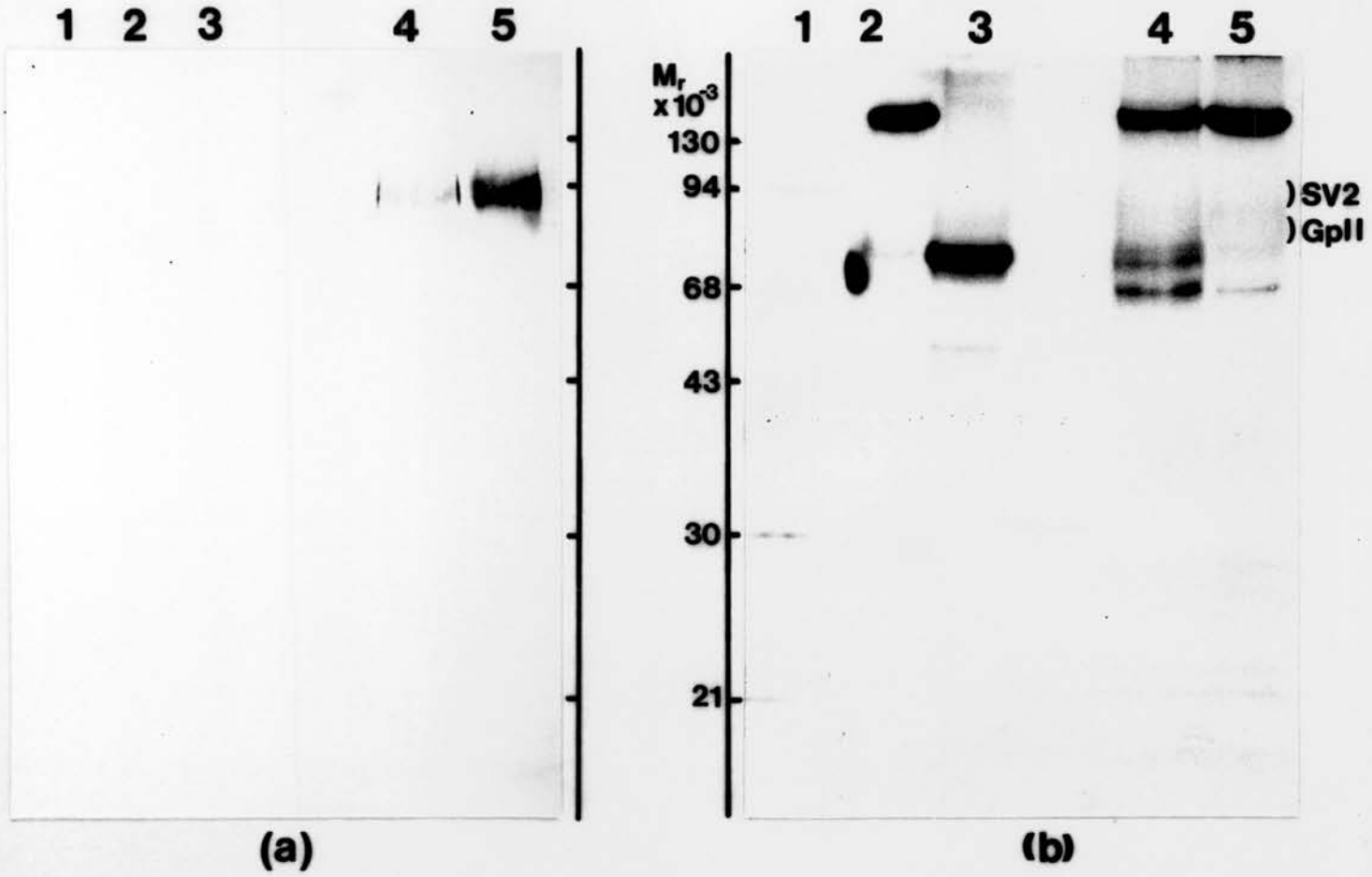
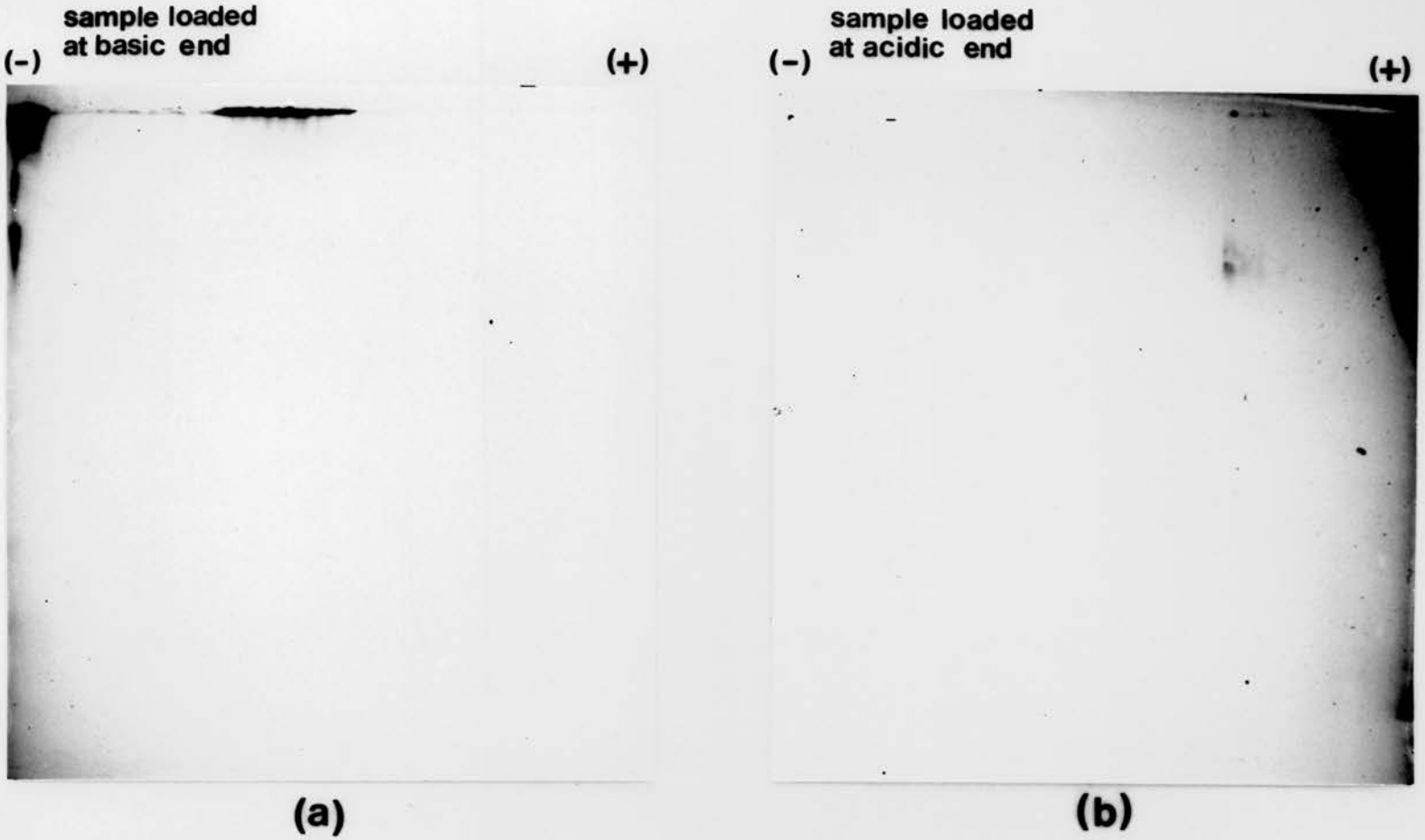


**Figure 4.20.**



second dimension properly (not shown).

When fractions from a solubilisation and phase separation of chromaffin granule membrane proteins in Triton X114 were probed with the SV2 monoclonal antibody its antigen was concentrated in the detergent enriched fraction, but was also present in significant amounts in the aqueous phase (Figure 4.21). The SV2-antigen behaved in this experiment like many other chromaffin granule glycoproteins which show similar distributions. The absence of the SV2-antigen from the phospholipid-rich phase however allows us to conclude that it is not related to glycoprotein IV nor to the polypeptides of the proton translocating ATPase, both found exclusively in this fraction (see Chapter 5).

The distribution of the SV2-antigen in the adrenal medullary microsomal fractions was analysed and shown to be concentrated in chromaffin granule membranes, with relatively minor amounts present in all three microsomal fractions (Figure 4.22). There was no indication that the nonglycosylated precursor to SV2, a polypeptide of $M_r$65,000, identified in PC12 cells after tunicamycin treatment (Buckley and Kelly, 1985), was present in these fractions. That the SV2-antigen was confined to only adrenal medullary tissue and was absent from adrenocortical tissue was demonstrated by immunofluorescence studies with thin tissue sections of adrenal glands (Dr. J. Haywood, unpublished); adrenal cortex and exocrine pancreatic tissue showed only background fluorescence levels.

Finally like SV2 the monoclonals to rat brain p65 and synaptophysin were also identified as minor components of chromaffin membrane preparations not previously identified by electrophoretic analysis (Phillips and Pryde, 1986; J.G. Pryde, S.L. Wood, and J.H. Phillips unpublished).

**Figure 4.21. Distribution of the SV2-Antigen After Solubilisation and Phase Separation of Chromaffin Granule membranes with Triton X-114.**

An 8-15% non-reducing gel; each track was loaded with 100µg of protein: track 1, whole chromaffin granule membranes; track 2, phospholipid-rich phase; track 3, detergent-rich phase; track 4, aqueous phase.

**Figure 4.22. The Distribution of the Sv2-Antigen in Adrenal Medullary Microsomal Membrane Fractions.**

An 8-15% non-reducing gel; each track was loaded with 100µg of protein: track 1, chromaffin granule membranes; track 2, Golgi-membranes; track 3, RER membranes; track 4 plasma membranes. Note: this blot was slightly overdeveloped and shows some non-specific binding by SAM-HRP.

Figure 4.21.

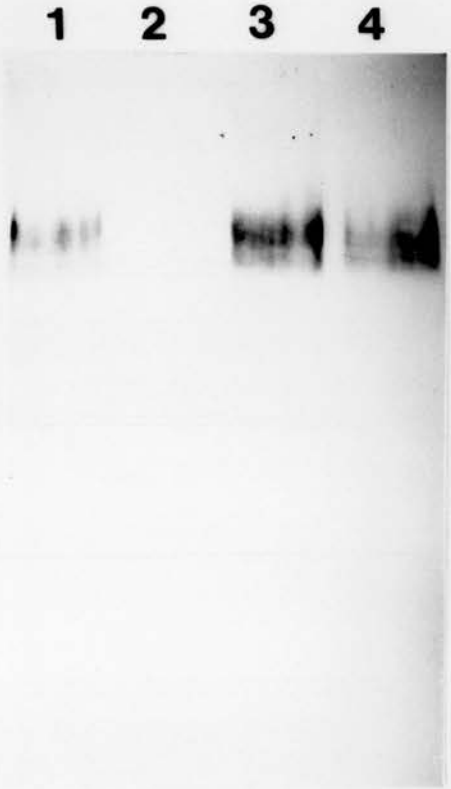



Figure 4.22.

DISCUSSION.

Antibodies against two relatively abundant chromaffin granule integral membrane proteins, cytochrome $b_{561}$ and DBH, and against the major secretory protein chromogranin A, have been used to characterise their distribution throughout subcellular fractions of adrenal medullary tissue. One of the main aims was to study the biogenic pathway involved in the processing of these membrane proteins. While they could be identified by antibody decoration in fractions enriched in markers for RER and Golgi membranes the polypeptides appeared to migrate identically to the mature and fully modified species. That the mature proteins can be identified immunologically in all fractions to varying degrees suggests either that they are present as artefacts of subcellular fractionation, for example, contamination by granule membranes, or that the precursors are indistinguishable on these gels. The possibility of identifying precursors to membrane proteins by this approach was further diminished in the light of cellular synthesis studies with isolated chromaffin cells (Chapter 6) where it was found that only secretory proteins could be identified amongst the newly synthesised radiolabelled proteins. While precursors to secretory proteins were identified by antibody screening, newly synthesised membrane proteins could not be found suggesting that they are present in very low concentrations. The radioiodination of membrane proteins and their immunoprecipitation is an approach which in some cases can provide the sensitivity required for identifying minor membrane components. However, purified cytochrome $b_{561}$ failed to iodinate efficiently and immunoprecipitation of the purified cytochrome was in addition very inefficient (Kilpatrick, 1985) and so that this approach could not be exploited.

Both the Coomassie staining and lectin overlay studies have shown some overlap between the subcellular fractions isolated. However, these approaches have also shown marked differences between the fractions. The three microsomal fractions have been tentatively identified as enriched in RER (Band III), Golgi membranes (Band II) and a plasma membrane fraction (Band I). Plasma membrane distribution on sucrose gradients is very heterogeneous and the acetylcholinesterase activity measured in Band II suggests that this fraction may be heavily contaminated by a plasma membrane fraction. The distribution of this enzyme is controversial however, since it may well redistribute during tissue disruption; in the adrenal medulla it has been reported from several subcellular fractions, including secretory granules (Burgun et al., 1985).

Similarities in the polypeptide patterns between microsomal Bands I and II, especially among the high $M_r$ glycoproteins, tend to suggest a close relationship exists between these fractions. The presence of the $M_r$ 32,000 protein which avidly binds lentil lectin is however characteristic of Band I, being almost completely absent from the Golgi fraction (Band II). Band I may represent a particular domain of the plasma membrane having a low protein to phospholipid ratio, in view of its distinctively low buoyant density. This is born out to some extent by the relatively simple polypeptide pattern of this fraction. A glycoprotein with an $M_r$ of 32,000 has been reported in previous adrenal medullary plasma membrane preparations (Wilson and Kirshner, 1976; Zinder et al., 1978), and domain-specific glycoproteins have been identified in rat hepatocyte plasma membrane preparations (Bartles et al., 1985).

Glycoproteins.

The distibution of many of the glycoproteins which show a characteristic "herring-bone" pattern on two-dimensional analysis appears to be widespread. Glycoprotein II (GpII) and GpIV appear enriched in the Golgi membrane fraction. Both these glycoproteins, although present in chromaffin granule membrane preparations, have not yet been shown to be uniquely restricted to these membranes. The two-dimensional gel analysis suggests that glycoproteins H,J, and K on the other hand are enriched in the granule membranes; this has been confirmed by density gradient centrifugation of adrenal medullary tissue homogenates whose fractions were probed with polyclonal antisera (Wood et al., 1985)

The possibility that precursor proteins to GpIIb and GpIV were present in the RER fractions was particularly striking (Figure 4.18d). These glycoproteins had collapsed into spots which suggest the absence of terminal sialic acid residues, normally added in the Golgi complex; no equivalent concanavalin A-binding glycoproteins were evident in the other subcellular fractions. The molecular weight heterogeneity shown by these two concanavalin A-binding proteins suggests that they have a substantial content of high mannose core oligosaccharides. If these are indeed the same polypeptides that are also identified in Golgi membrane and granule membrane enriched fractions they may be candidates for structural proteins, or proteins carrying out a function common to all the endomembranes. Thus the RER equivalent of GpIV, for example, will not leave the RER since RER membrane proteins apparently do not contain the terminal sugar residues added on in the Golgi complex. The relationship between these polypeptides must however wait for specific antibodies to answer such speculations and to identify

cellular locations unambiguously. The possibility that some of these glycoproteins may be of lysosomal origin has not been addressed. As yet adrenal medullary lysosomal membranes have not been isolated and so no electrophoretic characterisation is available.

Finally it has been shown that three membrane glycoproteins, in particular the SV2-antigen (Buckley and Kelly, 1985), are present in chromaffin granule membrane preparations. They cannot be identified with proteins previously identified by Coomassie staining or lectin overlay analysis. If these glycoproteins are present in the chromaffin granule membrane they are minor components. However, in the light of their widespread occurrence in neuro-endocrine tissues their further study may be of importance to the understanding of the membrane structure and function in relation to both secretion and biogenesis. The chromaffin granule membrane will therefore be a convenient source of this antigen.

**Subcellular Distribution of Defined Granule Antigens.**

Dopamine $\beta$-hydroxylase appeared to be an ideal candidate for following the biogenesis of an integral membrane protein which is exposed exclusively to the granule matrix. However, the anomalous behaviour of this protein when analysed by two-dimensional electrophoresis prevented an analysis of any subtle changes in relative molecular mass or isoelectric point.

Does each organelle posses its own unique set of proteins and are certain proteins shared?

The Golgi complex is such a dynamic organelle that it may in practice not be possible to identify in isolation its unique membrane components as can be done for secretory granule membranes.

The Golgi complex is continually receiving membrane from the RER, discharging membrane from its tran-cisternae and receiving membrane for recycling from the plasma membrane, so a priori this organelle can be expected to contain a mixture of endomembrane proteins. The electrophoretic analysis presented here suggests that this may in fact be the case, even if, in addition it contains its own unique enzymes and other proteins.

The only successful system for studying the biogenesis of membrane proteins has been the expression of viral coat proteins in cultured cell lines (Green et al., 1981). These studies show that like secretory proteins, integral membrane proteins traverse the Golgi complex. That membrane proteins could not be detected by immunoblotting and lectin overlay analysis of adrenal medullary membrane fractions, suggests that endogenous precursors may be present in only very small amounts; this is consistent with the idea that membrane proteins are extensively reutilised after exocytosis. In this context both cytochrome $b_{561}$ and dopamine $\beta$-hydroxylase have been identified in clathrin coated vesicles isolated from adrenal medullary tissue (M. Geisow, unpublished).

# CHAPTER FIVE

# FRACTIONATION OF MEMBRANE PROTEINS BY TEMPERATURE-INDUCED PHASE SEPARATION IN TRITON X-114: APPLICATION TO SUBCELLULAR FRACTIONS OF THE ADRENAL MEDULLA.

## Introduction.

### Analysis of Secretory Granule Fractions.

Secretory granule membrane fractions have been purified from many endocrine and exocrine tissues by differential and density gradient centrifugation. Analysis of their membrane proteins by polyacrylamide gel electrophoresis has revealed very complex polypeptide compositions. Interpretation of these polypeptide 'maps' is difficult even though such analysis has been improved by the introduction of two-dimensional electrophoretic separations and the transfer of polypeptides to sheets of nitrocellulose has enhanced the sensitivity of antibody and lectin overlays.

There are a number of well-known problems associated with the analysis of membrane proteins: (i) in many cases it is difficult to obtain pure membrane fractions, (ii) there is always contamination from adsorbed proteins of extracellular, cytoplasmic and matrix or luminal origin; (iii) the degree of this contamination depends upon the washing procedures used to remove the adsorbing soluble proteins, and can be minimised by washing with chaotropic agents, sodium cabonate (high pH) or low concentrations of detergents, however these procedures tend to lead to the loss of biological activities, (iv) no simple method exists to identify integral membrane proteins in the presence of such contamination.

Two-dimensional electrophoretograms of chromaffin granule membrane preparations are dominated by soluble proteins which contaminate these fractions, particularly as many integral membrane glycoproteins stain poorly with Coomasssie Blue. This latter problem has been overcome to some extent by staining polyacrylamide gels for carbohydrate with periodate-Schiff reagent (Huber et al., 1979) by radiolabelling membrane proteins, or by probing for

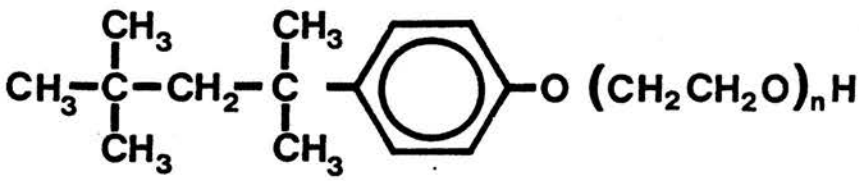
glycoproteins with radiolabelled or fluorescein-conjugated lectins (Abbs and Phillips, 1980; Cahill and Morris, 1979). The sensitivity of these techniques is improved if the separated polypeptides are electrophoretically transferred to cellulose nitrate sheets before decoration (Glass et al., 1981; Hawkes, 1982). The resolving power of electrophoretic techniques would be greatly increased however, if integral membrane proteins could first be separated into different families free from contamination by adhering soluble proteins.

**Phase Separation of Proteins in Triton X-114.**

Integral membrane proteins are by definition proteins which have hydrophobic domains that anchor them in the lipid bilayer. Nonionic detergents with polyoxyethylene head groups can displace and replace much of the normal lipid environment around these proteins, while hydrophilic proteins bind very little detergent (Helenius and Simons, 1975). The members of the Triton X series of detergents (Figure 5.1; n 6) form small micelles (140 molecules for Triton X-100) when dispersed in water above their critical micelle concentrations at $0^{o}C$. However, at higher temperatures the micelle size increases, and depending upon the number of hydrophilic oxyethylene moieties on the detergent, the micellar aggregates which form come out of solution, turning it turbid at a characteristic temperature known as the cloud point. For Triton X-100 (n=9-10), this is at $64^{o}C$. For Triton X-114 (n=7-8), however, the cloud point is at $20^{o}C$ (Helenius and Simons, 1975; Bordier, 1981).

Bordier (1981) took advantage of both the differential binding of Triton X-114 to integral membrane proteins, and the formation of a detergent-rich phase when Triton X-114 is warmed to temperatures above its cloud point, to develop a simple and rapid method for the

Figure 5.1. Polyoxyethylene p-t octylphenol: the Triton X Series of Detergents.

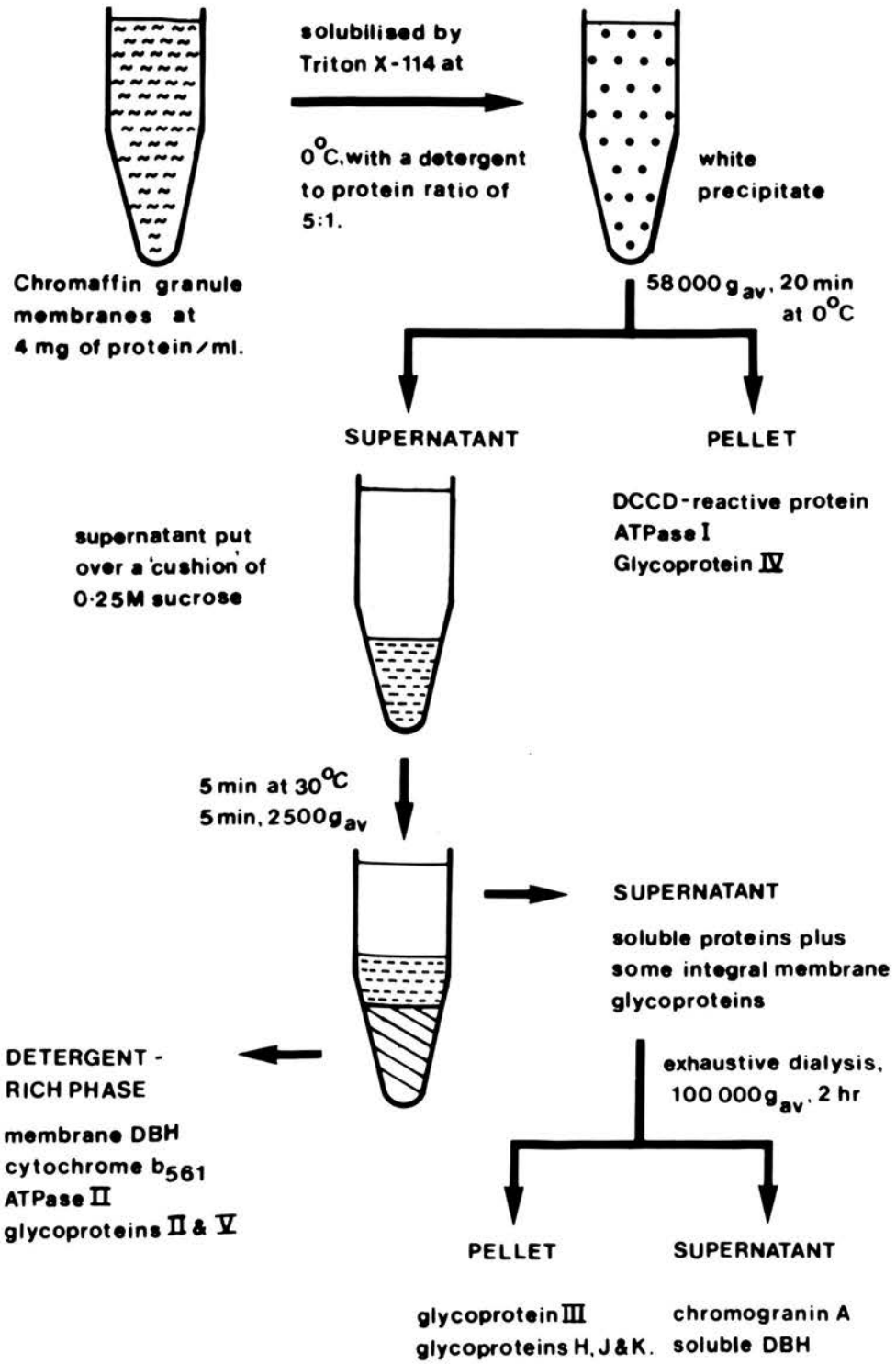


n is the average number of ethylene oxide units per molecule.

separation of membrane proteins. He solubilised a membrane preparation at $0^{o}C$, warmed it at $30^{o}C$, then recovered the detergent-rich phase after a low speed centrifugation. Integral membrane proteins such as acetylcholinesterase, bacteriorhodopsin and cytochrome c oxidase were recovered from the detergent-rich phase while hydrophilic proteins (serum albumin, cytochrome c) remained in the aqueous phase.

This chapter describes how Triton X-114 was used to solubilise the membrane proteins of subcellular fractions of the adrenal medulla and to separate them by phase partioning. The chromaffin granule membrane was used as the model for development of the phase separation protocol. This is summarised in Figure 5.2 which shows that two families of integral membrane proteins can be identified and separated from those integral membrane proteins found as anticipated in the detergent-rich phase; they appear to differ in their degree of hydrophobicity. All these fractions are free of the contamination from extrgranular and soluble matrix proteins that have dominated two-dimensional electrophoretograms of earlier chromaffin granule membrane analyses.

Figure 5.2. Solubilisation and Phase Separation of Chromaffin Granule Membrane Proteins in Triton X-114.



Removal of Adherent Soluble Protein.

A two-dimensional electrophoretic separation of chromaffin granule membrane proteins is shown in Figure 5.3a. Several major proteins are labelled. A distinct subset of proteins, almost identical to those released from granules by hyposmotic shock, is removed by washing the membranes with 0.1M $Na_2CO_3$ which breaks vesicular structures and removes adherent soluble protein (Howell and Palade, 1982; Higgins, 1984) (Figure 5.3b). The majority of these polypeptides are of chromaffin granule matrix origin. They have previously been characterised by immunological techniques (Fischer-Colbrie and Frischenschlager, 1985) and shown to contain the chromogranins A and B, enkephalin precursors and a small amount of soluble dopamine $\beta$-hydroxylase. There are however, a few non-matrix proteins (shown in Figure 5.3b) which are almost certainly adhering to the cytosolic surface of the chromaffin granule membrane; among these are the $\beta$-subunit of the mitochondrial $F_1$-ATPase ($M_r$51,000, pI5.1, which was identified by decoration with an antiserum to yeast $F_1$-ATPase) and a triplet of proteins ($M_r$68,000, pI5.8) components identified on gels of Golgi membrane enriched fractions (see Figure 4.8c). After two or three cycles of washing, depletion of these components is almost complete; a trace of chromogranin A remains as the most conspicuous contaminant. Having identified this set of proteins in this way, the membranes were not normally prewashed with $Na_2CO_3$ before the phase separation.

**Figure 5.3. Two-Dimensional Electrophoretic Analysis of Chromaffin Granule Membrane Proteins.**

(a). Chromaffin granule membrane proteins (300ug); these membranes have not been washed in sodium carbonate.

(b). Proteins (300µg) removed from granule membranes by washing with sodium carbonate.

Proteins here have been separated on 8-15% gels under reducing conditions and stained with Coomassie blue following focusing.

The arrows in (b) indicate those proteins not identified in the analysis of chromaffin granule matrix proteins shown in Figure 4.4; a protein $M_r$ 68,000 found concentrated in Golgifractions; $\beta$-subunit of the mitochondrial $F_1$ ATPase and calmodulin (identified by co-migration with the purified protein and by shifts in $M_r$ in the presence of calcium ions. The major proteins indicated are: dopamine $\beta$-hydroxylase, DBH; chromogranin A, CgA; glycoprotein III of Huber *et al.* (1979) and cytochrome $b_{561}$, CYT. The standards in the second dimension are described in the legend to Figure 4.1.

**Figure 5.4. Phase Separation of Chromaffin Granule Membrane Proteins.**

(a). Proteins from each of the Triton X-114 phases were separated on an 8-15% one-dimensional gel under reducing conditions and stained with Coomassie blue. Each track contains 100µg of protein: track 1, standard proteins as described in Figure 4.1; track 2, whole chromaffin granule membranes; track 3, phospholipid-rich phase; track 4, detergent-rich phase; track 5, aqueous phase; track 6, chromaffin granule matrix proteins in which the major band is chromogranin A ($M_r$ 70,000).

(b). A two-dimensional analysis of the proteins (300µg) in the phospholipid-rich phase (track 3 above).

The dicyclohexylcarbodiimide- reactive protein, identified by labelling membranes with [$^{14}$C]-DCCD is indicated. The major Coomassie staining spots are subunits of the proton translocating ATPase (Percy and Apps, 1986).

Figure 5.3.

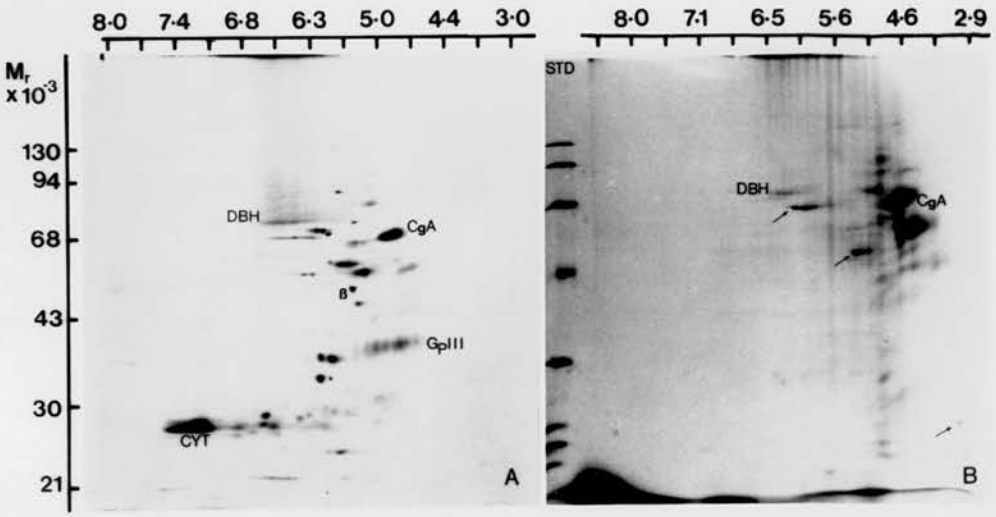
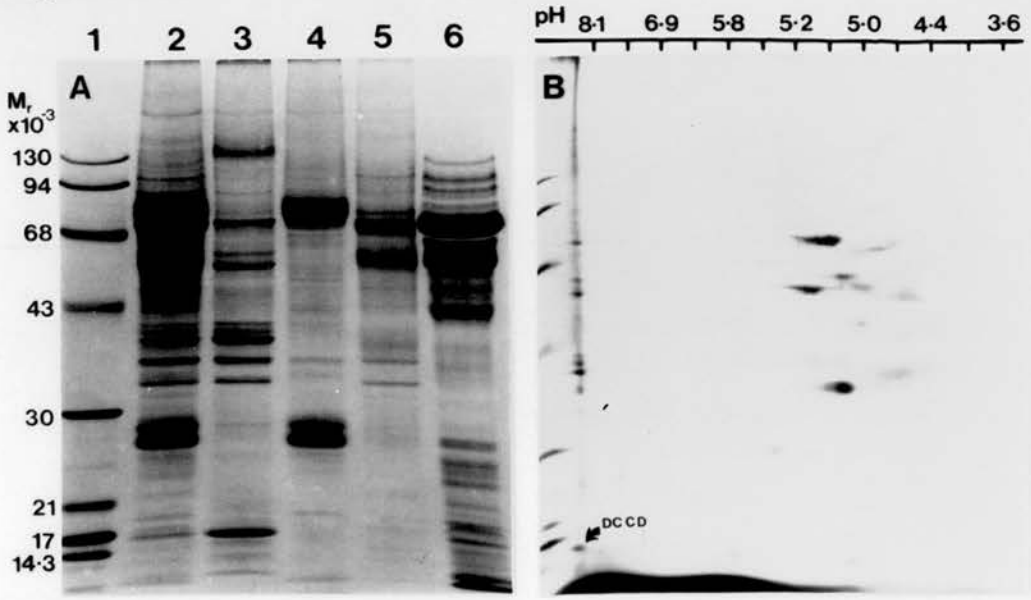



Figure 5.4.



**Solubilisation Phase Separation in Triton X-114.**

Although rich in cholesterol and phospholipids chromaffin granule membranes were apparently fully solubilised by Triton X-114 when used at a detergent to protein ratio of 5:1 at $0^{o}C$. However, unlike solubilisation with Triton X-100 or octaethyleneglycol dodecylether (Apps et al., 1980a), Triton X-114 fails to maintain all the membrane components in solution. This effect was not pH dependent and the precipitate that forms is probably composed of large mixed micelles (possibly multilayered structures) enriched in phospholipids (especially sphingomyelin) and cholesterol (see below), and some very hydrophobic proteins (approximately 10% of the chromaffin granule membrane protein). This precipitate, unlike the detergent-rich or aqueous phases, showed an enrichment in the ratio of phospholipid to protein and was therefore referred to as the "phospholipid-rich phase".

On warming at $30^{o}C$, the supernatant became turbid as large micellar aggregates formed (Bordier, 1981). The detergent-rich phase was separated from an aqueous phase by low speed centrifugation through sucrose; it contained about 50% of the membrane protein, with 30-40% in the aqueous supernatant.

When samples of the three phases were examined by one-dimensional polyacrylamide gel electrophoresis (Figure 5.4), each phase was found to contain a quite distinct family of proteins; Coomassie staining showed little overlap between them. This is summarised in Table 5.1 which shows the distribution and relative molecular mass of 24 of the major proteins associated with chromaffin granule membrane fractions. The three phases were then analysed by two-dimensional electrophoresis, identifying proteins where possible by immunological methods or by lectin decoration.

Table 5.1. The Distribution of Major Chromaffin Granule Membrane Polypeptides.

| $Mr \times 10^{-3}$ | Phase | | | Identification |
|---|---|---|---|---|
| | Phospholipid | Detergent | Aqeous | |
| 104 | | | + | matrix |
| 93 | | | + | Matrix |
| 78 | | + | + | dopamine |
| 75 | | + | | β-hydroxylase |
| 72 | + | | | ATPaseI |
| 70 | | | + | chromogranin A |
| 68 | | | + | chromogranin A |
| 65 | | + | | |
| 62 | | + | | matrix |
| 58 | +/- | | + | |
| 56 | | | + | matrix |
| 53 | + | | | |
| 50 | | + | | |
| 47 | + | | | |
| 40 | + | | | |
| 39 | + | | | |
| 37 | + | | | |
| 34 | + | +/- | +/- | |
| 31 | + | | +/- | |
| 26 | | + | | cytochrome $b_{561}$ |
| 24 | | + | | cytochrome $b_{561}$ |
| 17 | + | | | |
| 16 | + | | | DCCD-reactive |
| 13 | + | | | |

Identification based on immume replicas, labelling with [$^{14}$C]DCCD, and previous work on matrix components (Apps et al., 1985). +, major location;+/-, minor location.

Phospholipid-rich Phase.

A two-dimensional electrophoretogram of the proteins in the phospholipid-rich phase is shown in Figure 5.4b. Not all the polypeptides identified by one-dimensional analysis (Bands 1-6) can be identified, due either to their basic nature or to poor focusing, a problem commonly found with very hydrophobic proteins. An example of this is a very hydrophobic polypeptide with an apparent molecular mass of 16,000 (shown in Figure 5.4b) that can be covalently labelled with dicyclohexylcarbodiimide (Sutton and Apps, 1981); it is one of the components of the proton-translocating ATPase of the membrane (ATPase I) (Apps et al., 1982; Apps et al., 1983). The DCCD-reactive protein and other components of this enzyme complex which are found in this fraction are discussed below (see also Percy et al., 1985).

This fraction may appear to contain only hydrophobic polypeptides. However, it may also contain polypeptide complexes, such as the proton-translocating ATPase, in which some relatively hydrophilic polypeptides may be separated as part of a complex. The diffuse band ($M_r$140,000) in Figure 5.4a (track 3) is an aggregate, and it can be removed by heating the sample at 100°C in the absence of mercaptoethanol before electrophoresis. This fraction also contains a number of membrane glycoproteins which are analysed below.

Lipid Analysis of Triton X-114 Phases.

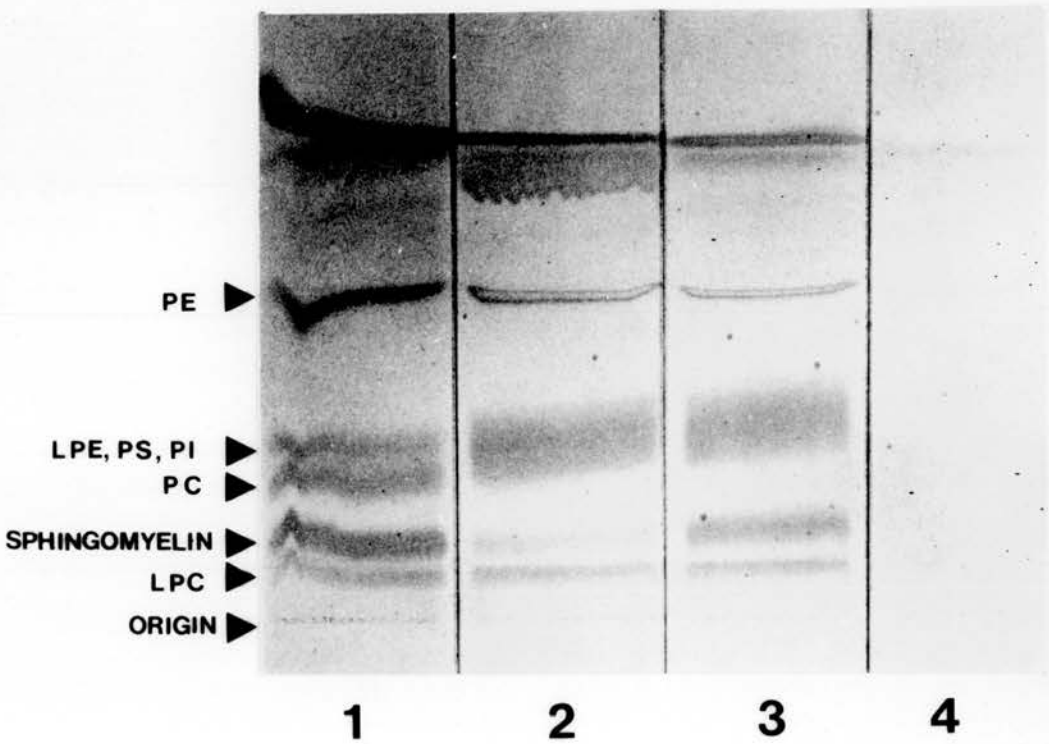
Total lipid phosphorus for the chromaffin granule membrane was 1.5μmol/mg of protein. Making the assumption that the ratio of soluble protein to granule membrane protein is 5:1 the total granule value is thus 0.3μmol of lipid phosphorus/mg of protein, in

agreement with previously reported estimates (Winkler, 1976; Trifaro et al., 1967; Trifaro and Dworkind, 1971). Higher membrane lipid phosphorus values (up to 2.4μmol/mg of protein) have been reported (Winkler et al., 1970; Schneider, 1972; Da Prada et al., 1972; Phillips, 1973), however, these differing values may reflect the washing procedures used to remove adhering soluble proteins. If the soluble protein remaining in the aqueous phase following dialysis to precipitate integral membrane proteins is subtracted from the total membrane protein, a value for lipid phosphorus in the membrane of 2.2μmol/mg of protein can be calculated.

The phospholipid to protein ratio was greatest in the precipitate produced after solubilisation of the membranes; 5.5μmol of lipid phosphorus/mg of protein. This represents a 3.7-fold increase over the uncorrected (for adhering soluble protein) membrane value. The detergent-enriched phase showed no enrichment in phopholipid (1.9μmol/mg) over the membrane value. No lipid phosphorus was detected in the aqueous phase. The initial precipitate was therefore operationally defined as the "phospholipid-rich" phase. Although there was an equal distribution of total phospholipid between the "phospholipid-rich" and the detergent-rich phases, a striking observation was that 95% of the sphingomyelin was recovered in the phospholipid-rich phase (see Figure 5.5). Phosphatidylethanolamine on the other hand was enriched to some degree in the detergent-rich phase.

Cholesterol analysis showed that the cholesterol/protein ratio was typically 4 times higher in the phospholipid-rich phase (5.3μmol cholesterol/mg protein) than that estimated for the chromaffin granule membrane (1.2μmol/mg protein). No cholesterol was detected in the aqueous phase and there was no enrichment of cholesterol in

Figure 5.5. Phospholipid Analysis of the Triton X-114 Phases.




Separation of chromaffin granule membrane phospholipids by thin layer chromatography (see Chapter 2, page **75** for details). Track 1, phospholipid-rich phase; track 2, detergent-rich phase; track 3, whole chromaffin granule membranes; track 4, aqueous phase. Phosphatidyl ethanolamine, PE; lysophosphatidyl ethanolamine, LPE; phosphatidyl serine, PS; phosphatidyl inositol, PI; phosphatidyl choline, PC; lysophosphatidyl choline, LPC.

the detergent phase over that measured for the whole membranes.

The phospholipid-rich phase contained 0.5-0.6% (w/v) Triton X-114; the detergent-rich phase contained about 9%, and the aqueous phase about 0.06%.

Detergent-rich Phase.

This phase presents a strikingly simple polypeptide pattern after one-dimensional polyacrylamide gel electrophoresis and staining with Coomassie Blue (Figure 5.4, Track 4). It is dominated by the amphiphilic form of dopamine $\beta$-hydroxylase and by cytochrome $b_{561}$, the two most abundant proteins of the membrane. Together they may represent over 40% of the total protein associated with the membrane preparation (Winkler and Westhead, 1980). Cytochrome $b_{561}$, a transmembrane protein (Abbs and Phillips 1980; Duong and Fleming, 1984), gives this phase its highly distinctive red colour.

The presence of the majority of dopamine $\beta$-hydroxylase in the same phase as the hydrophobic integral membrane protein cytochrome $b_{561}$ was rather surprising. As a glycoprotein it was predicted that DBH would partition like a number of other membrane glycoproteins had (see below), into the aqueous phase. However, most of the enzyme partitioned into the detergent-rich phase. Some was in the aqueous phase and remained there even after removal of residual detergent by dialysis (see below); this was consistent with the dual location of DBH in chromaffin granules.

Aqueous Phase.

Two-dimensional gels of the aqueous phase revealed three categories of proteins: 1. contaminating matrix proteins; 2. unidentified hydrophilic proteins, most likely arising from the

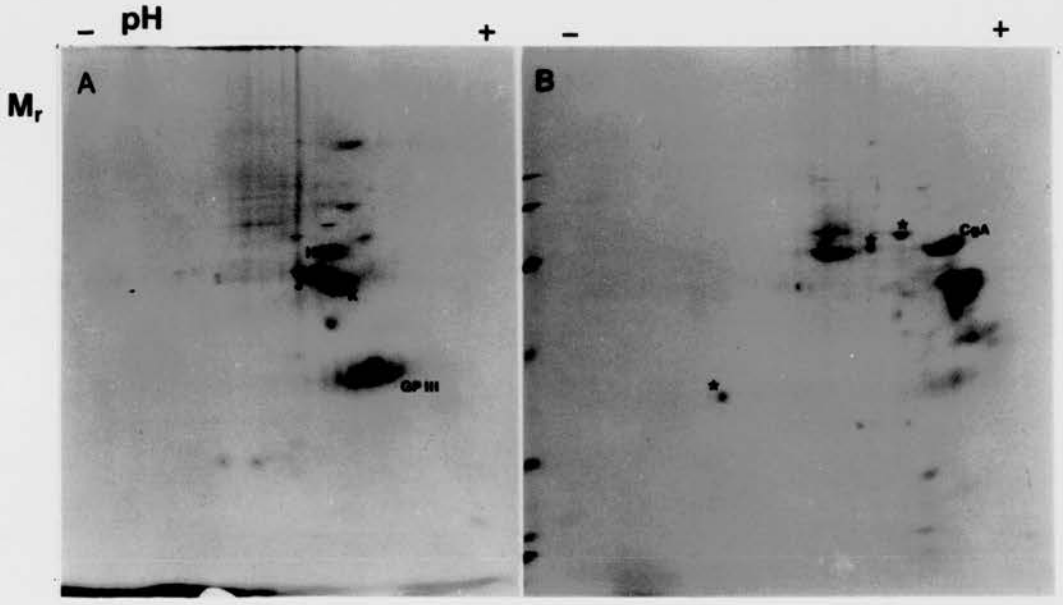
cytosol, other organelles, or from an extracellular location and 3. several proteins known to be glycosylated, and which are regarded as genuine membrane constituents.

The aqueous phase contained a residue of Triton X-114; 0.06% (w/v), as found by analysis (Garewal, 1973). More than 90% of this could be removed by extensive dialysis against buffer containing Amberlite XAD-2. About 20% of the protein in this fraction was precipitated during this dialysis and could be recovered by high speed centrifugation. A two-dimensional electrophoretogram of this material (Figure 5.6a) revealed several proteins previously characterised as membrane components (Huber et al., 1979; Fischer-Colbrie et al., 1982; Gavine et al., 1984). Proteins in categories (1) and (2) above remained in solution (Figure 5.6b) and are essentially the same as those removed from the membranes by $Na_2CO_3$ washing (Figure 5.3b), although one or two may be candidates for genuine peripheral membrane proteins. Dialysis is therefore a simple procedure for recovery of membrane glycoproteins from this fraction.

Chromaffin Granule Membrane Glycoproteins.

Chromaffin granule membrane glycoproteins were revealed by decoration with radioiodinated lectins after transfer to nitrocellulose. Figure 5.7 shows one-dimensional autoradiograms of membranes after solubilisation and phase separation in Triton X-114, and their decoration with radioiodinated (a) concanavalin A (b) lentil lectin and (c) wheat germ agglutinin. The one-dimensional autoradiogram of proteins decorated with [$^{125}$I]-concanavalin A is very complex, but does demonstrate that each of the Triton fractions (Figure 5.7; tracks 3-5) contains many glycoproteins. The

Figure 5.6. Chromaffin Granule Membrane Proteins in the Aqueous Phase.




Two-dimensional electrophoretogram stained with Coomassie blue.
(a). Proteins (100µg) precipitated by the removal of residual detergent from the aqueous phase by dialysis (details Chapter 2 page 59); the chromaffin granule membrane glycoproteins are enriched to a degree where they show appreciable staining with Coomassie blue.

(b). Proteins remaining in solution following the removal of residual detergent from the aqueous phase by dialysis.

Standard proteins are those described in Figure 4.1. Chromogranin A (CgA).

Figure 5.7. [$^{125}$I]-Lectin Overlays of Triton X-114 Phases.

Chromaffin granule membranes were solubilised and phase separated as shown in Figure 5.3, which is an identical Coomassie stained gel of the ones shown in this figure. After transfer to nitrocellulose the proteins were decorated with radioiodinated lectins as described in Chapter 2 (page 65).

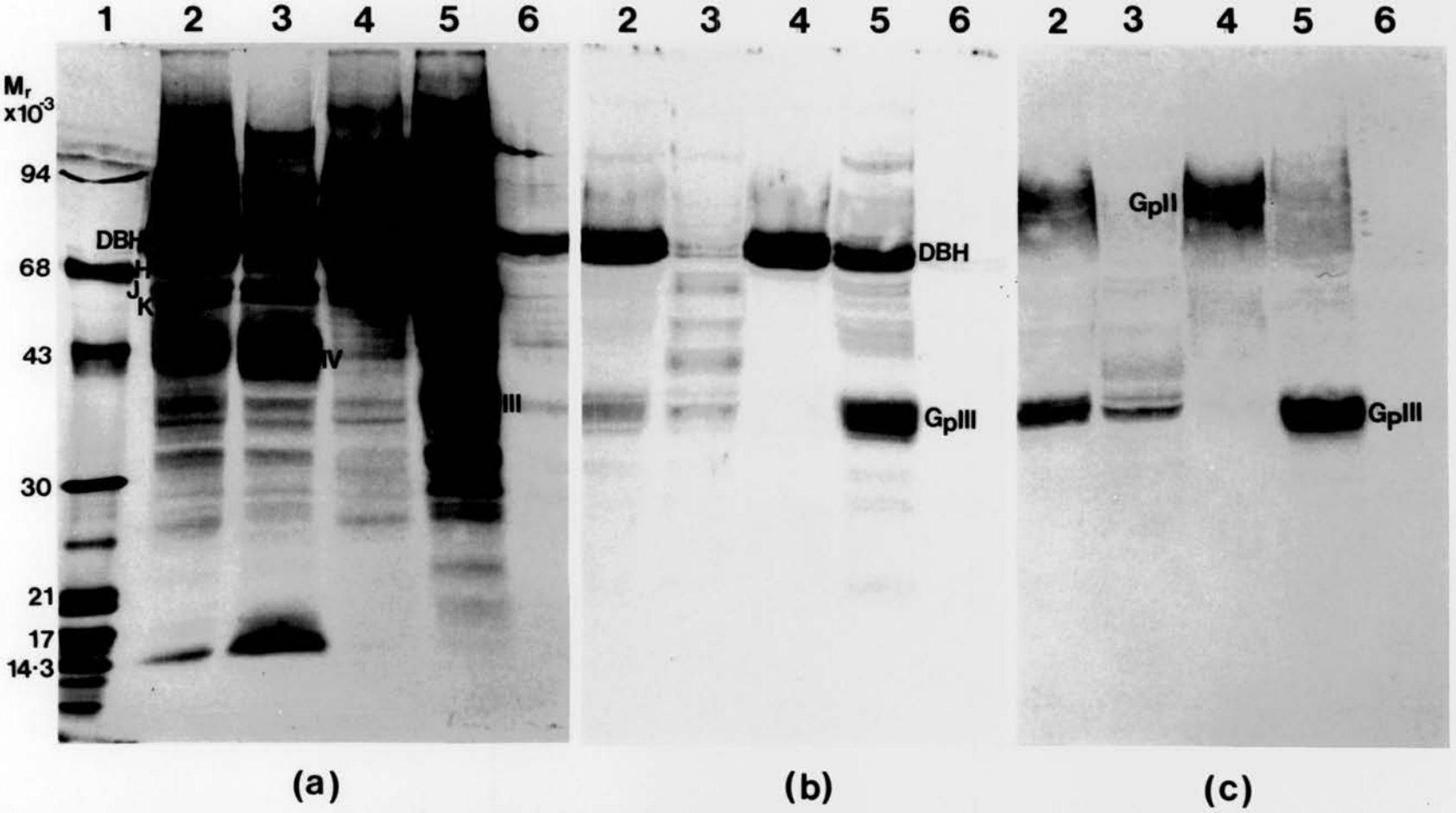
(a) [$^{125}$I]-concanavalin A. Track 1, standard proteins as described in Figure 4.1; track 2, whole chromaffin granule membranes; track 3, phospholipid-rich phase; track 4, detergent-rich phase; track 5, aqueous phase; track 6, chromaffin granule matrix proteins.

(b) [$^{125}$I]-Lentil lectin; identity of the tracks as in (a) above.

(c) [$^{125}$I]-Wheat germ agglutinin; identity of the tracks as in (a) above.

The distribution of the concanavalin A binding glycoproteins is shown in Table 5.2. A full description of the glycoprotein nomemclature is given in Figure 2 of Gavine et al. (1984) with which track 2 of this figure should be compared. Dopamine β-hydroxylase, DBH; glycoproteins H,J&K, H,J&K; glycoprotein III, GpIII; glycoprotein IV, GpIV.

Figure 5.7.

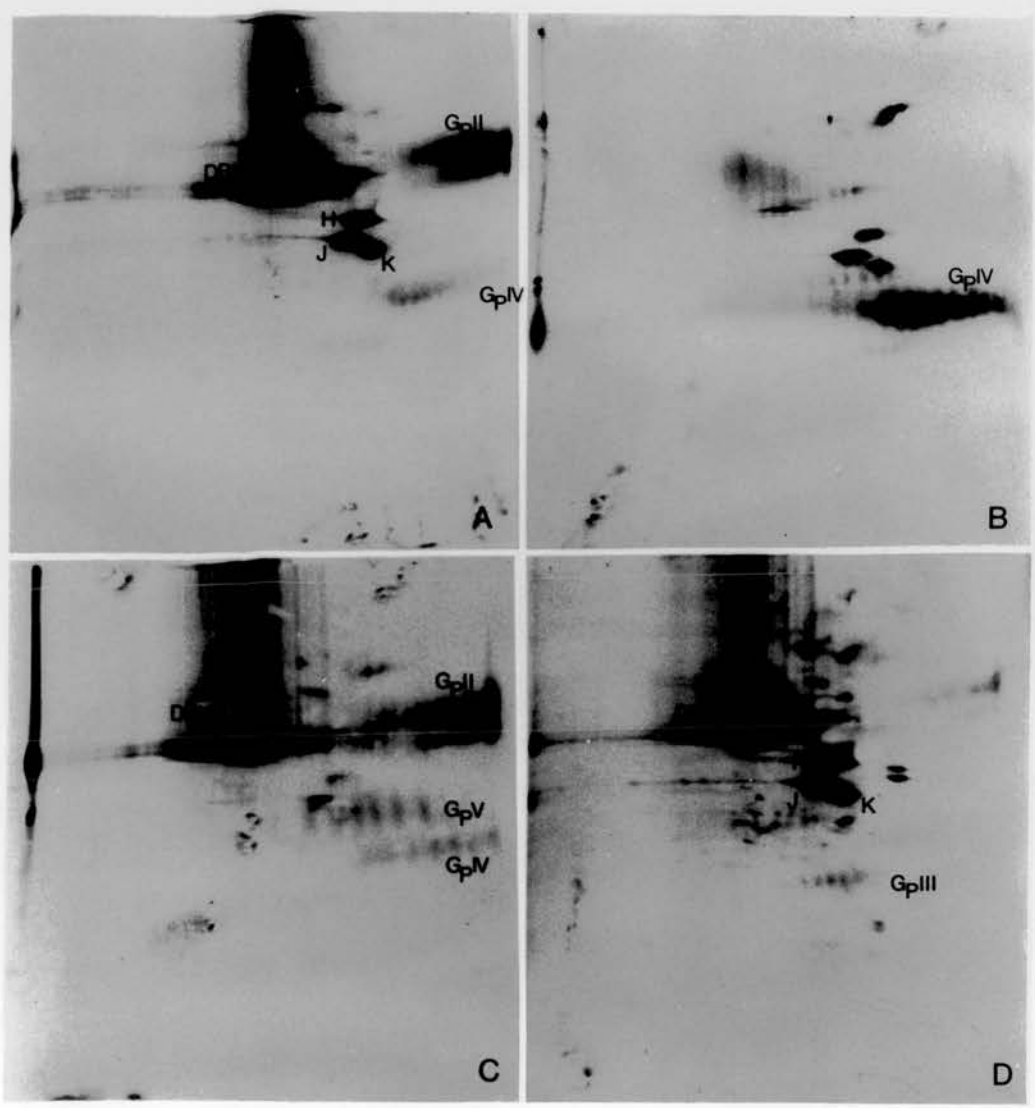

1 2 3 4 5 6
2 3 4 5 6
2 3 4 5 6
$M_r$ x$10^{-3}$
94
68
43
30
21
17
14·3
DBH
J
K
IV
III
DBH
GpIII
GpII
GpIII
(a)
(b)
(c)

[$^{125}$I]-lentil lectin is more discriminating however, and shows that DBH partitions into the detergent-rich phase (Figure 5.7b; compare tracks 3,4&5). This lectin also shows that glycoprotein III, an integral membrame glycoprotein, partitions almost exclusively into the aqueous fraction. This is also demonstrated by WGA binding (Figure 5.7c, track 5). WGA does not bind to DBH and is a useful tool for identifying glycoprotein II whose very diffuse staining pattern is almost exclusively localised to the detergent-rich fraction (Figure 5.7c, track 4).

All the chromaffin granule membrane glycoproteins appear to bind concanavalin A (Gavine et al., 1984), however, the complexity of the one-dimensional separation is better resolved by two-dimensional electrophoresis. Two-dimensional overlays of the three Triton fractions are shown in Figure 5.8 and a composite drawing of the lectin binding proteins in the various fractions is shown in Figure 5.9. In Figure 5.8a, an overlay of whole membranes is shown to give a qualitative impression of the relative intensity of concanavalin A-binding by the glycoproteins, before they become enriched in the various phases. Figures 5.8a and 5.8b may be compared with Figures 5.3a and 5.4b which have been stained with Coomassie blue.

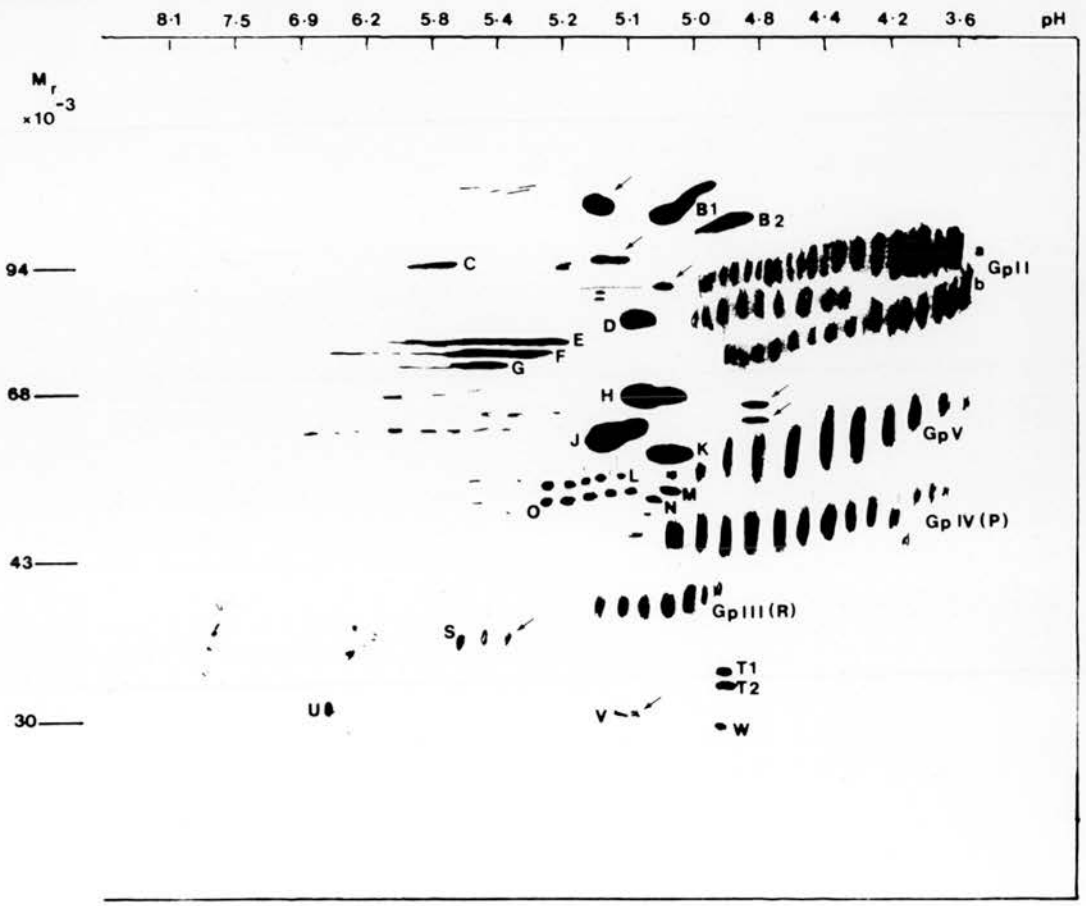
Figure 5.8 shows that two major categories of glycoprotein can be identified; some focus as a series of spots spread over a wide range of isoelectric points in the acid region, while others show little isoelectricfocusing heterogeneity. In at least some cases the former category results from the heterogeneity of the sialylation of complex oligosaccharides. In general, the three phases contain distinct glycoproteins and their distribution is summarised in Table 5.2..

Figure 5.8. [$^{125}$I]-Concanavalin A Overlays of Two-Dimensional Gels of Triton X-114 Phases.




Chromaffin granule membranes and the Triton fractions (300µg of protein) were lyophilised then solubilised for IEF under reducing conditions. The second dimension is an 8-15% gel. After blotting onto nitrocellulose the sheets were incubated with radioiodinated lectins as described in Chapter 2 (page **66**).
(a). Chromaffin granule membranes.
(b). Phospholipid-rich phase.
(c). Detergent-rich phase.
(d). Aqueous phase.

Figure 5.9. Composite Drawing of the [$^{125}$I]-Concanavalin A Binding Proteins of the Chromaffin Granule Membrane.




Arrows denote components that remain in solution after dialysis of the aqueous phase. Labelling follows the nomenclatures of Gavine et al. (1984) and Huber et al. (1979).

Table 5.2. The Distribution of Major Chromaffin Granule Membrane Glycoproteins.

| Designation | | Phase | | |
|---|---|---|---|---|
| Gavine et al. (1984) | Huber et al. (1979) | Phospho--lipid | Detergent | Dialysis Pellet |
| $B_1$ | | + | | + |
| $B_2$ | | | + | |
| C | | | + | |
| | GpII | | + | |
| D | | | | + |
| E | GpI | | + | |
| F | GpI | | + | |
| G | GpI | | + | |
| H | | + | | + |
| J | | + | | + |
| K | | + | | + |
| | GpV | | + | |
| L | | + | | + |
| M | | | | + |
| N | | | | + |
| O | | | | + |
| P | GpIV | + | | |
| R | GpIII | | | + |
| $T_1$ | | | | + |
| $T_2$ | | | | + |
| U | | | | + |
| W | | | | + |

The labelling of components shown in Figure 5.5, follows the nomenclature of Huber et al. (1979) and Gavine et al. (1984); GpV has not been named previously. E, F and G are the components of dopamine $\beta$-hydroxylase, as revealed by antibody binding.

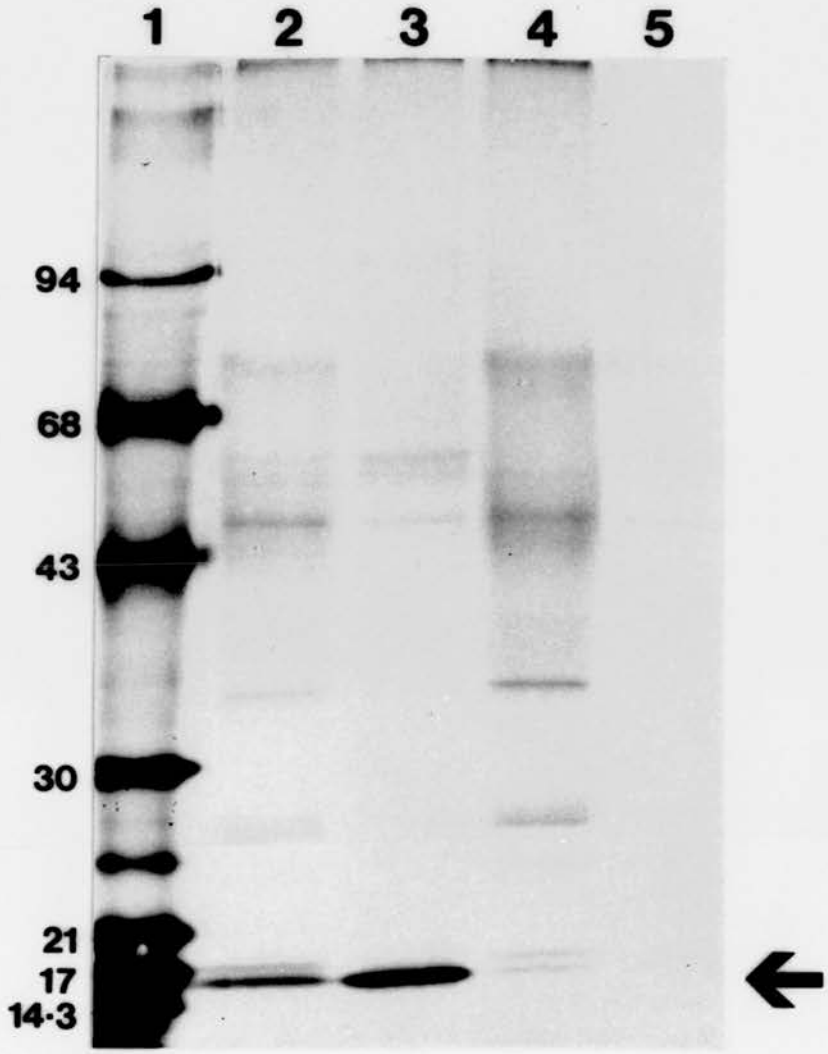
Distribution of ATPases.

The phospholipid-rich phase contains the proton-translocating ATPase (ATPase I) activity of chromaffin granule membranes, 2- to 3-fold enriched relative to intact membrane (Percy et al., 1985). The one-dimensional electrophoretogram shown in Figure 5.3 (track 3) identifies six major Coomassie staining polypeptides in this ATPase I fraction. These bands (designated 1-6; Percy et al., 1985) have relative molecular masses of 140,000, 72,000, 57,000, 40,000, 34,000 and 17,000 (see also Table 5.1). This polypeptide pattern was identical to that shown by a similar phospholipid-rich fraction produced as a by-product of the purification of cytochrome $b_{561}$ (Apps et al., 1980 ; Pryde, 1982).

Band 6 was identified as the hydrophobic [$^{14}$C]DCCD-reactive protein ($M_r$16,000; see Sutton and Apps, 1981), by labelling with [$^{14}$C]DCCD. Figure 5.10 shows a fluorogram of labelled chromaffin granule membrane proteins after solubilisation and phase separation in Triton. This fluorogram can be compared with an identical Coomassie stained electrophoretogram shown in Figure 5.3a. The only band showing a significant amount of labelling is band 6. This protein phase separates along with ATPase I activity and both are excluded from the detergent-rich phase and from the aqueous phase.

Chromaffin granule membrane preparations are contaminated with polypeptides of the mitochondrial $F_1F_0$-ATPase (Apps and Schatz, 1979; Apps et al., 1983; Cidon and Nelson, 1983; Cidon et al., 1983). The distribution of the subunits of the catalytic complex of this enzyme were analysed by immunoblotting with an antiserum from rabbits immunised with bovine heart mitochondrial $F_1$-ATPase (Apps and Schatz, 1979). Figure 5.11a (track 2) shows a one-dimensional separation of chromaffin granule membranes and a two-dimensional

Figure5.10. Phase Distribution of the Chromaffin Granule Membrane DCCD-reactive Protein.




A fluorograph of a one-dimensional separation of chromaffin granule membranes and Triton X-114 solubilised and phase separarted proteins following labelling of the membranes with [$^{14}$C]-DCCD. Track 1, [$^{14}$C]-formaldehyde-labelled protein standards (see Figure 4.1); track 2, whole chromaffin granule membranes; track 3, phospholipid-rich phase; track 4, detergent-rich phase; track 5, aqueous phase.

The arrow indicates the position of the proton translocating ATPase DCCD-reactive protein.

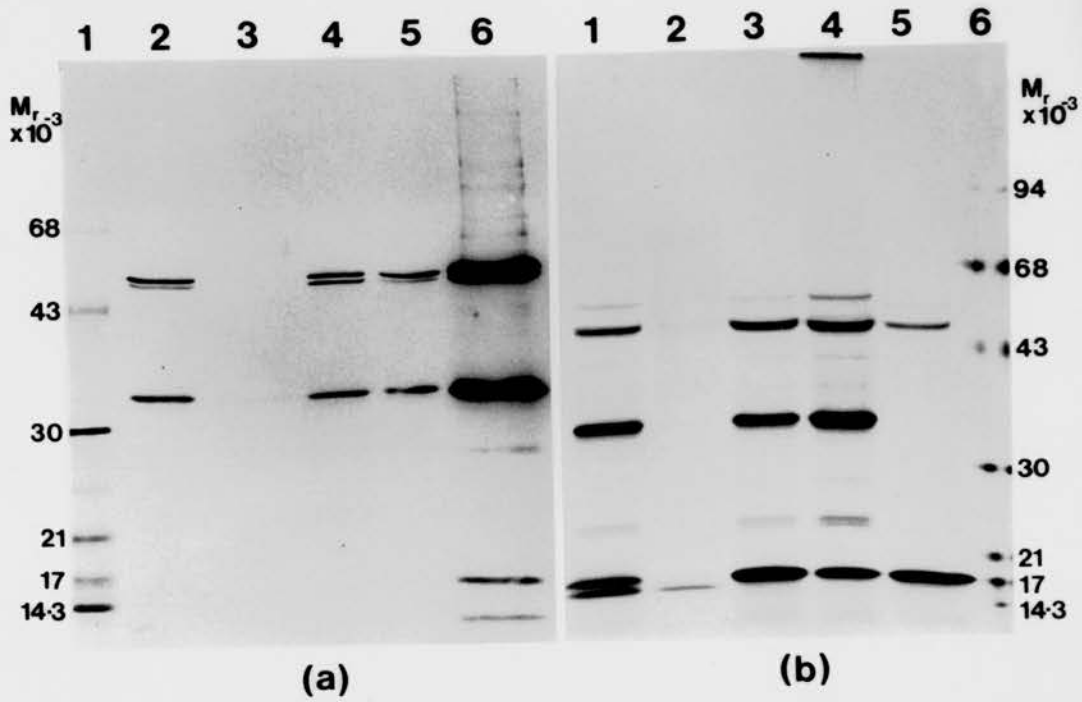
Figure 5.11. Phase Distribution of the Mitochodrial $F_1F_0$-ATPase Polypeptides.

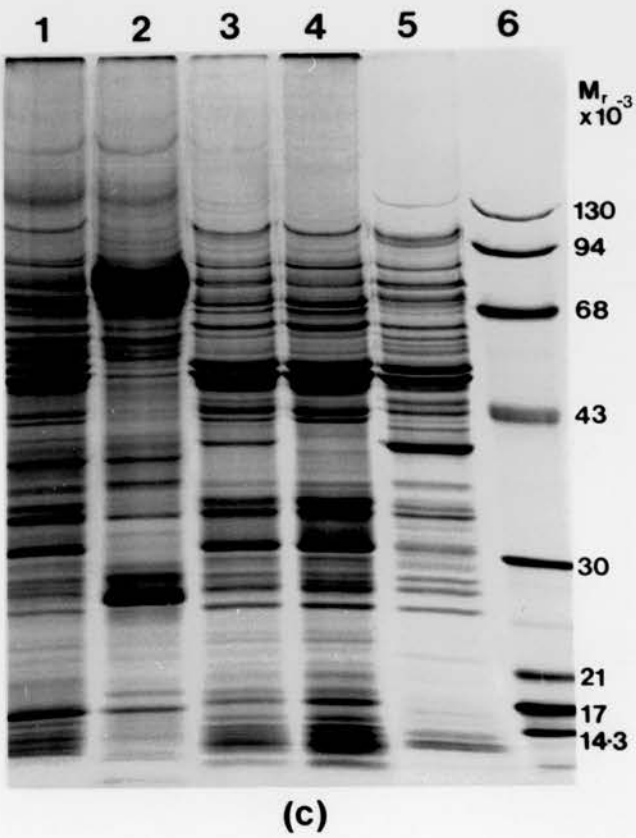
(a). An immune blot of an 8-15% one-dimensional electrophoretogram of chromaffin granule membrane proteins decorated with an antiserum to mitochondrial $F_1F_0$-ATPase. Track 1, standard proteins (as shown in Figure 4.1); track 2, whole chromaffin granule membranes; track 3, phospholipid-rich phase; track 4, detergent-rich phase; track 5, aqueous phase; track 6, purified bovine heart mitochondrial $F_1$-ATPase.

(b). Fluorogram of mitochondrial membrane proteins labelled with N,N'-dicyclohexyl[$^{14}$C]-carbodiimide. Track 1, a mixture of the phospholipid-rich phase proteins (75µg) from chromaffin granule membranes with the detergent-rich phase proteins (75µg) from mitochondrial membranes in order to demonstrate the difference in the electrophoretic mobilities of their $F_0$-ATPase DCCD-reactive proteins; track 2, whole chromaffin granule membranes; track 3, whole mitochondrial membranes; track 4, detergent-rich phase of mitochondrial membranes; track 5, aqueous phase; track 6, standard proteins.

(c). The Coomassie stained electrophoretogram of the mitochondrial membrane proteins shown in (b) above. Each track was loaded with 150µg of protein and samples were reduced with 0.5% mercaptoethanol.

Figure 5.11.


1 2 3 4 5 6
1 2 3 4 5 6
$M_r$ x10$^{-3}$
68
43
30
21
17
14·3
$M_r$ x10$^{-3}$
94
68
43
30
21
17
14·3
(a)
(b)

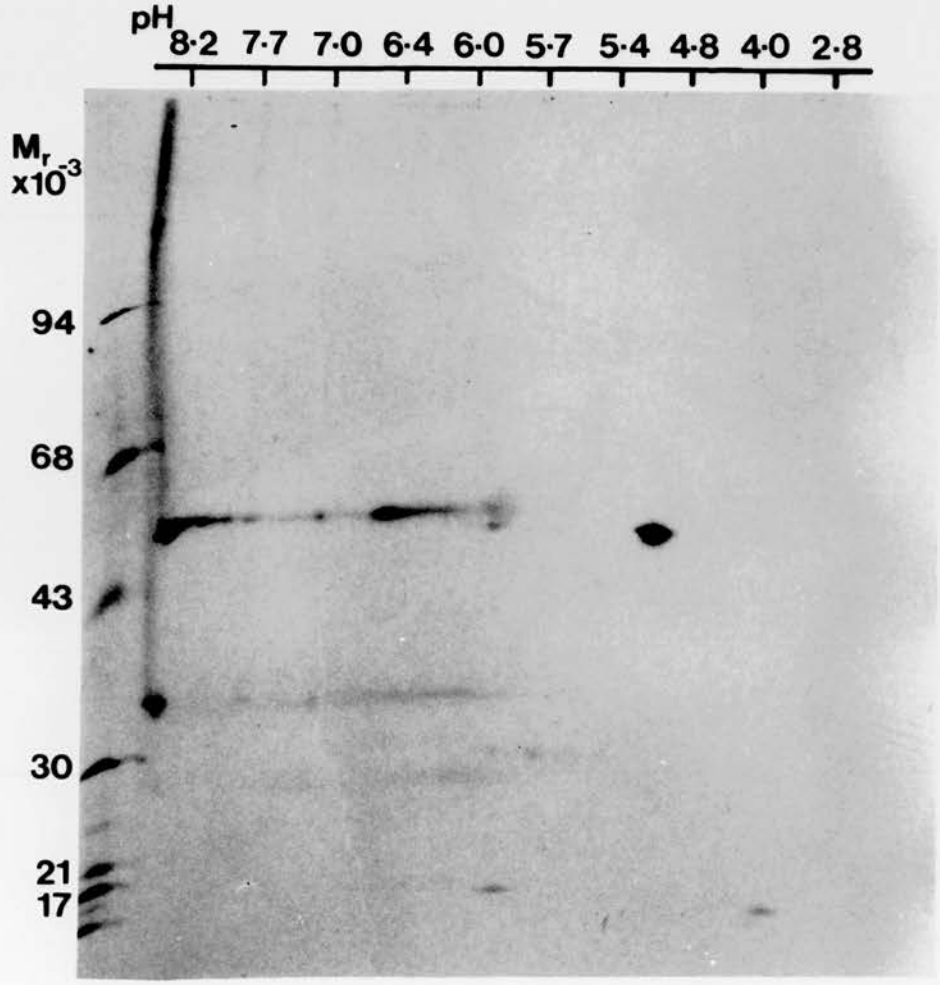

1 2 3 4 5 6
$M_r$ x10$^{-3}$
130
94
68
43
30
21
17
14·3
(c)

immune blot of this track is shown in Figure 5.12, showing the $\beta$-subunit of the ATPase ($M_r$50,000; pI5) with the more basic $\alpha$-subunit focusing poorly above it. These mitochondrial enzyme subunits are confined to both the detergent-rich and aqueous phase as is shown in tracks 4&5 of Figure 5.11a. The distribution of the mitochondrial membrane DCCD-reactive proteins is shown in Figure 5.11b. Tracks 3,4&5 of this figure may be compared with identical Coomassie stained tracks shown in Figure 5.11c (tracks 2,3&4). The DCCD-reactive polypeptide of the mitochondrial $F_1F_0$-ATPase has an apparent relative molecular mass of 17,000, but is actually much smaller as determined by gene sequencing.

Mitochondrial membranes when solubilised with Triton X-114 do not produce the distinctive phospholipid-rich fraction. The DCCD-reactive polypeptide of the mitochondrial $F_1F_0$-ATPase is, unlike its chromaffin granule membrane counterpart, distributed between both the detergent and aqueous phases as shown in Figure 5.11b. That the chromaffin granule membranes are contaminated with the mitochondrial DCCD-reactive protein is shown in Figure 5.10 (tracks 2,4&5). The relative molecular masses of the proton translocating ATPase DCCD-reactive proteins from these sources are different (Sutton and Apps, 1981) as is shown in Figure 5.11b (track 1) where samples of the chromaffin granule phospholipid-rich phase and the mitochondrial detergent-rich phase, in which their respective low-$M_r$ DCCD-reactive polypeptides are enriched, were mixed.

The identity of the other mitochondrial membrane DCCD-reactive polypeptides shown in Figure 5.11b is not known. However, that of $M_r$50,000 may be the $\beta$-subunit of the $F_1$-ATPase (Satre et al., 1982). The polypeptide of $M_r$34,000 which partitions exclusively

Figure 5.12. Two-Dimensional Immune Blot of Chromaffin Granule Membranes With Anti-$F_1$-ATPase Antiserum.




After IEF proteins were separated on a 7-15% gel. The nitrocellulose replica was incubated with anti $F_1$-ATPase antiserum diluted 1:100 then decorated with radioiodinated protein A. The autoradiogram above shows a major immune reactive spot $M_r$ 50,000 pI5 which is the -subunit of the mitochondrial $F_1$-ATPase.

into the detergent-rich phase may be the mitochondrial phosphate transporter (Houstek et al., 1981); this can also be identified as a major Coomassie staining band in Figure 5.4a.

**Application to other Subcellular Fraction.**

How applicable is the Triton X-114 separation technique to other membranes?

In Chapter 4 we saw that our knowlege of the integral membrane protein components of adrenal medullary organelles other than the secretory granules was only fragmentary. Solubilisation and phase separation in Triton X-114 when applied to a mitochondrial membrane fraction and to the three microsomal sub-fractions (Figure 4.3) enabled the identification and comparison of the major integral membrane protein of these fractions.

Figure 4.3a, the phase separation of Golgi membranes (Track 2), showed that like the secretory granule membrane these membranes produce a phospholipid-rich phase (Track 3) in addition to detergent-rich and aqueous phases. If this track is compared with that from granule membranes in Figure 5.4 (track 3) there are a number of polypeptides which appear to be common to both fractions. Band 2 and 3 ($M_r$ 72,000 and 57,000) are the major bands in the Golgi membranes with the latter predominating. The 72,000 polypetide is the major ATPase subunit (Percy and Apps, 1986). However, in contrast to the granule membranes there does not appear to be a significant amount of the DCCD-reactive protein (band 6, $M_r$16,000) in the Golgi-enriched fraction and as the Golgi membrane detergent-rich fraction contains relatively little cytochrome $b_{561}$ or DBH; this fraction does not appear to be derived from chromaffin granule contamination.

Discussion.

The Triton X-114 methods developed in this chapter provide a route for simplifying the analysis of proteins from any cellular membranes, by dividing them into four classes by their relative hydrophobicity. The most hydrophilic, or peripheral proteins, were removed by sodium carbonate washing, or were those remaining in the aqueous phase after detergent removal by dialysis; various glycoproteins were precipitated from the aqueous phase by detergent removal - they were thought to be transmembrane proteins with large oligosaccharide side chains; then in order of increasing hydrophobicity, are the proteins of the detergent-rich and phospholipid-rich phases. The technique is rapid and has the advantage of working under non-denaturing conditions.

Some glycoproteins, notably H,J and K appeared both in the aqueous phase and in the phospholipid-rich phase. This problem of the partitioning of integral membrane proteins into the aqueous phase was not encountered by Bordier (1981), but it has been noted after the phase partitioning of platelet proteins (Clemetson et al., 1984) and of acetylcholine receptor-rich membranes proteins of Torpedo californica (Maher and Singer, 1985). The aqueous phase was found to contain 700μM Triton X-114, 3-4 times the critical micelle concentration of the detergent, and presumably sufficient to mask some hydrophobic domains on these proteins. Most glycoproteins distributed characteristically into a single phase however (Figure 5.7).

The glycoproteins are listed in Table 5.2. Those given Gp designations are in general acidic and highly heterogeneous. This presumably arises from sialylation, as has been demonstrated directly for GpII by analysis (17μmol per 100mg of protein;

Fischer-Colbrie et al., 1982) or by neuraminidase treatment (GpIII; Gavine et al., 1984).

Some other features of these experiments which are of general interest are discussed in Pryde and Phillips (1986) and Pryde (1986).

# CHAPTER SIX

## Cellular Synthesis of Bovine Adrenal Medullary Secretory Proteins.

## Introduction.

Until recently little work had been done on the biogenesis of the major secretory proteins of the adrenal medulla, the chromogranins and enkephalins. Early studies using perfusion of whole glands with [$^{3}$H]leucine (Winkler et al., 1971; 1972) suggested that newly synthesised chromogranins were passed through a pool of immature granules, the whole process from synthesis to mature granule taking about 48 hours (Baumgartner et al., 1974). More recently the biogenesis of these proteins has been investigated by in vitro translation and processing (Kilpatrick et al., 1983; Falkensammer et al., 1985a&b; Fischer-Colbrie et al., 1986); together with cDNA cloning and sequencing, this has provided a secure framework for investigations of chromogranin A, and sequences of other relevant proteins will no doubt be known within the next two or three years.

## Chromogranins.

A cDNA clone for chromogranin A has recently been sequenced (Benedum et al., 1986). It reveals a polypeptide of 431 amino acids proceeded by a signal sequence of 18 amino acids. The unmodified polypeptide thus has molecular weight of 48,000, although it migrates on SDS gels at a position corresponding to an $M_r$ of 70,000. This anomalous migration may be a consequence of clusters of glutamic acid residues in the sequence, although the protein itself is not particulary acidic (pI 4.8, see Figure 4.4).

The sequence contains eight pairs of basic amino acid residues representing potential proteolytic cleavage sites. Six of these are located in the C-terminal 120 amino acids of the sequence, and cleavage here is probably responsible for the formation of the

chromogranin A family of proteins found within chromaffin granules, and known to be produced post-translationally (Kilpatrick et al., 1983), although this has not yet been unequivocally demonstrated by sequencing.

Just over 5.4% of the relative molecular mass of chromogranin A is carbohydrate (Kiang et al., 1982). The oligosaccharides are exclusively O-linked, and contain N-acetylgalactosamine, galactose and sialic acid (both N-acetyl- and N-glycolylneuraminic acid). The protein does not therefore bind concanavalin A. Binding sites for the galactose-specific lectin PNA are only revealed after neuraminidase treatment (Apps et al., 1985).

Chromogranin B migrates on gels with a higher relative molecular mass ($M_r$100,000) and more basic pI (5.2) than chromogranin A. It also appears to give rise to a family of polypeptides by proteolysis. Little is known of its glycosylation, but decoration of two-dimensional gels shows binding of concanavalin A and Pisum sativum agglutinin (both specific for mannose and glucose), and binding of PNA (galactose-specific) after neuraminidase treatment (Apps et al., 1985). The chromogranin B polypeptides are also sulphated, probably on tyrosine (Rosa and Zanini, 1981; Rosa et al., 1985a), and these proteins appear to be identical to a family identified in the anterior pituitary called secretogranin I (Rosa et al., 1985b)

A third protein family (chromogranin C) has been identified immunologically, apparently the same as the pituitary protein secretogranin II (Rosa and Zanini, 1983). The parent protein has $M_r$86,000 and pI5. It is not particularly abundant in the bovine adrenal medulla, but is more abundant in other species (Fischer-Colbrie et al., 1986).

**Cell-Free Synthesis of the Chromogranins.**

Messenger-RNA has been isolated from bovine adrenal medullae and translated in either reticulocyte lysate or wheat germ translation systems in the presence of radiolabelled amino acids (Kilpatrick et al., 1983; Falkensammer et al., 1985). The radiolabelled polypeptide products were immunoprecipitated with antisera raised against the mature chromogranin A and B families, and the primary translation products analysed by two-dimensional polyacrylamide gel electrophoresis. The primary translation product of chromogranin A was a doublet of spots, $M_r$ 73,000 and 75,000 with pI's 5.3 & 5.2 respectively (Falkensammer et al., 1985; Kilpatrick, 1985), while chromogranin B, was a single spot with an $M_r$ of 98,000, pI 5.6. The isoelectric points of these precursors are more basic than their mature products.

These primary translation products each contain a signal peptide ($M_r$1,500 to 3,000) which is proteolytically removed when translations are carried out in the presence of microsomal preparations from the dog pancreas. After the removal of its signal peptide chromogranin B has an $M_r$ of 96,000; pI 5.7. The two precursors of chromogranin A are processed to a single polypeptide of $M_r$ 70,000. Why two primary translation products are apparently present is not known, although the presence of multiple primary translation products has been demonstrated for secretory protein I which is stored in the secretory granules of the parathyroid gland (Majzoub et al., 1982) and which has been shown to be almost identical to chromogranin A (Cohn et al., 1982; Kruggel et al., 1985). In the presence of microsomal fractions the four primary translation products of secretory protein I are converted into a single polypeptide $M_r$ 71,000 (Majzoub et al., 1982).

In this chapter I shall demonstrate that the proteolytically cleaved translation product undergoes no further post-translational modifications in the rough endoplasmic reticulum but that it has to be transported to the Golgi complex, a process which is energy dependent, to undergo O-glycosylation within this organelle.

**Biosynthesis of Dopamine β-hydroxylase.**

Dopamine β-hydroxylase is present in both a soluble form (sDBH) in the lysate and as a membrane-bound component (mDBH) (Duch et al., 1968). During secretion of catecholamines only the soluble form is released while the membrane-bound component is retained (Viveros et al., 1979). Early radiolabelling studies with perfused rat and bovine glands suggested that both forms of DBH were labelled at equal rates (Gagnon et al., 1976; Ledbetter et al., 1978). However, studies by Winkler et al. (1971, 1972) suggested that following secretion chromaffin granule membranes were reutilised, and that granule membrane proteins incorporated very little radioactivity during protein synthesis in pulse-labelling studies. These results suggested that the two forms of DBH may be synthesised independently. These two studies can be reconciled if it is assumed that the former study encountered problems with the adsorption of soluble granule matrix proteins to the membranes.

Are the two forms of DBH derived from a common precursor? It has been reported that newly synthesised mDBH can be converted into sDBH within the chromaffin granule (Helle et al., 1981; Sabban et al., 1983). However, there is also the possibility that sDBH may also arise from a failure to receive membrane targeting information during translation.

A monoclonal antibody cross-reacting with an epitope common to both forms of DBH has identified a polypeptide, $M_r$58,000, which is of low abundance in both granule lysates and purified membranes. It has been suggested that this polypeptide may be the primary translation product of DBH with its signal peptide removed: this would be produced in an *in vitro* translation system in the absence of microsomes. When DBH is chemically deglycosylated two polypeptides are produced with relative molecular masses of 67,000 and 58,000 (Nolan *et al.*, 1985). In another study using rat adrenal mRNA (Sabban and Goldstein, 1984) a primary translation product of $M_r$67,000 was identified in the absence of microsomal fractions. However, synthesis of DBH in cells (PC12) taken from a rat phaeochromocytoma (McHugh *et al.*, 1985) and in the presence of tunicamycin, an inhibitor of glycosylation which prevents the co-translational transfer of the high core mannose oligosaccharide to asparagine residues, showed synthesis of two polypeptides of $M_r$67,000 and 63,000. While the major product of cellular synthesis immunoprecipitated from cell lysates would be unlikely to carry the signal peptide, the translation product in the cell free system would be expected to have a higher molecular mass. Indeed, translation of bovine adrenal medullary mRNA in a wheat germ system has produced an unglycosylated precursor of 67,000 (Sabban and Goldstein 1984), while translation in a rabbit reticulocyte lysate on the other hand synthesised a primary product of $M_r$72,000 (Joh *et al.*, 1983).

The biosynthetic pathway to noradrenaline and adrenaline involves the activity of four enzymes (Blaschko, 1939). The final enzyme of the pathway, phenylethanolamine N-methyl transferase (PNMT), is not expressed in neurones, ganglia or adrenal cells which

only take synthesis as far as noradrenaline. The enzymes tyrosine hydroxylase, DBH and PNMT have been shown to have homologous sequences within their primary structures, suggesting that they are coded for by related genes, and may share common antigenic sites when they are present in the form of newly translated polypeptide chains prior to post-translational modification (Joh et al., 1983). There is therefore potentially a problem in studying DBH precursor polypeptides in immunoprecipitates produced by polyclonal antisera and it may be that monoclonal antibodies will be needed to tackle this problem.

Results.

## Chromaffin Cell Isolation.

Chromaffin tissue is surrounded by the adrenal cortex and treatment with collagenase is needed before these tissues can be separated. The crude adrenal medullary cell preparation is therefore heterogeneous, containing among other cell types cortical cells and fibroblasts. Successful isolation of large numbers of chromaffin cells depends on the quality of the batch of collagenase used. Collagenase preparations from many sources were tested and these gave cell yields (without purification on Percoll) of $20x10^6$ cells per gland. This yield compares favourably with cell numbers obtained in many of the early preparations reported by other laboratories (Fenwick et al., 1978). There are more recent reports however of yields of $10^8$ cells per gland. Such cell numbers are needed for efficient purification on Percoll gradients. A batch of collagenase (Clostridium histolyticum) from Boehringer eventually produced cell yields of greater than $10^8$ per gland and after purification on Percoll these preparations produced 40 to $50x10^6$ cells per gland. The experiments reported here are with cells purified on Percoll and where unpurified cell preparations have been used this is indicated in the text.

## Cell Morphology.

Figure 6.1a. shows a phase contrast micrograph of cells after isolation with collagenase and before purification on Percoll. There are a large number of phase bright cells about 20μm in diameter, many smaller cells about 10μm in diameter and erythrocytes. As an index of viability, cells were treated with trypan blue and greater than 90% excluded the dye. Cells were also

**Figure 6.1. Phase-Constrast and Fluorescence Micrographs of Freshly Isolated Chromaffin Cells.**

**(a).** Freshly isolated chromaffin cells in suspension culture. The major cell type is 20µm in diameter and has a prominent refractile ring. (Bar 50µm).

**(b).** Shows a fluorescence micrograph of cells shown in (a). Viable cells display bright green fluorescence while the nucleus of non-viable cells is accesible to the red fluorochrome ethidium bromide.

**(c).** A high power phase contrast micrograph and its companion fluoromicrograph **(d)** of freshly isolated chromaffin cells after purification on Percoll.

**Figure 6.2. Chromaffin Cells in Culture.**

Chromaffin cells in culture after (a) 18hr; (b)&(c) 3-4 days.

After overnight in culture the cells have only just begun to spread on the plastic surface and in many preparations cells aggregated into strings which appeared to hinder attachment and spreading. Providing cells were not seeded at high density they produced axon-like processes, characteristic of chromaffin cells in culture after a few days (6.2b). At high density contact inhibition prevented the production of processes (6.2c). Cell attachment and spreading was markedly improved if dishes were coated with rat-tail collagen (not shown).

Figure 6.1.

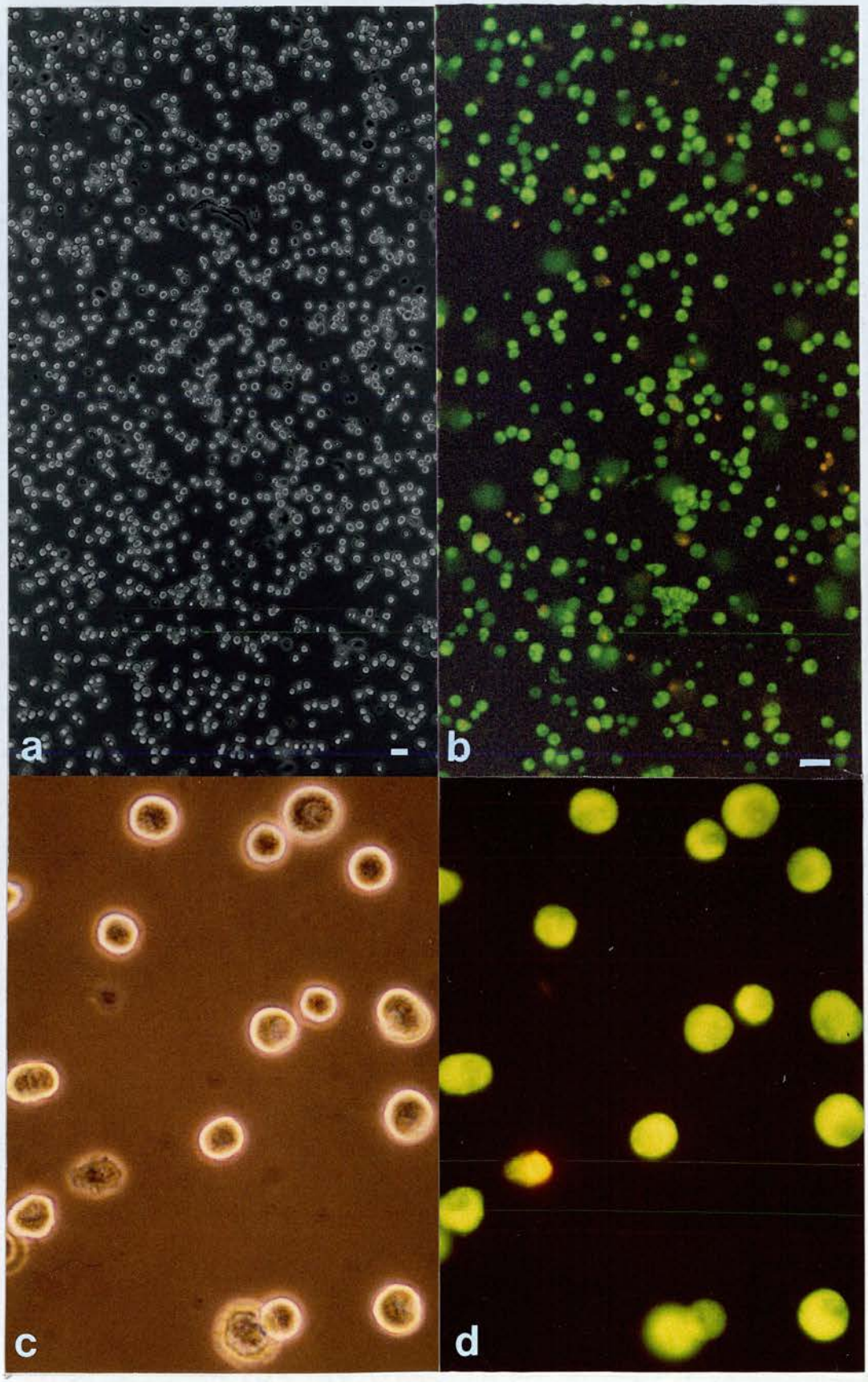


Figure 6.2.

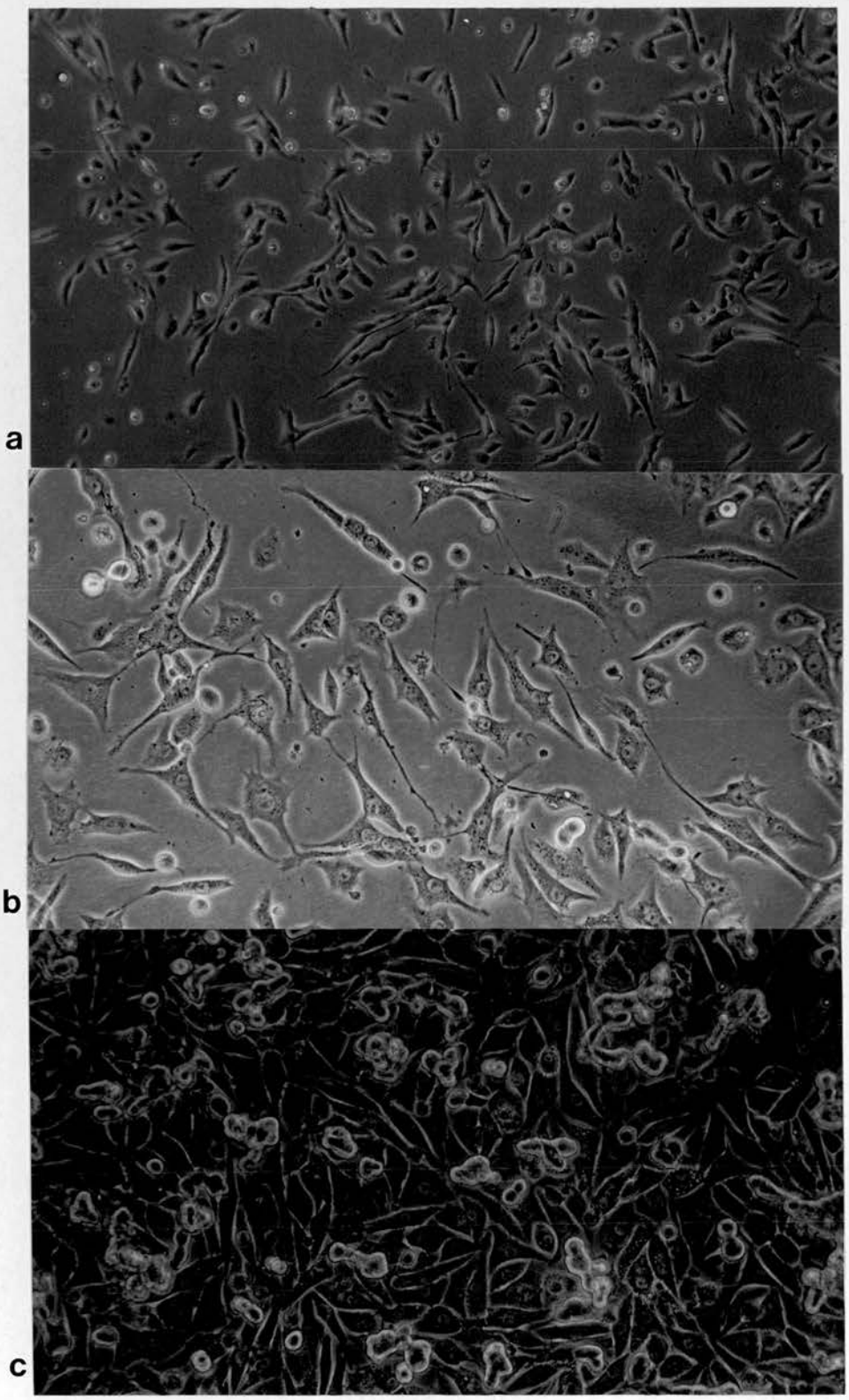


Table 6.1. Isolation of Chromaffin Cells.

| Cell fraction | Total Yield of Cells $\times 10^{-6}$ | Catecholamines Adrenaline (nmol/$10^6$ cells) | Noradrenaline (nmol/$10^6$ cells) | Adrenaline: Noradrenaline. |
|---|---|---|---|---|
| Crude Cells. | 344±160 (5) | 111±30 (5) | 32±13 (5) | 3.5 |
| Percoll Gradient:- | | | | |
| Debris. | - | 43±31 (3) | 53±22 (3) | 0.8 |
| Adrenocortical Cells. | 47±31 (5) | 91±58 (5) | 48±38 (5) | 1.9 |
| Chromaffin Cells. | 176±78 (5) | 148±73 (5) | 13±8 (5) | 11.4 |
| Erythrocytes. | - | 9 | 5 | 1.8 |

A crude cell suspension was isolated by perfusion of three glands in the presence of collagenase and fractionated by isopycnic centrifugation on Percoll. Values are mean ±S.D. ($\sigma$=n-1) of the number of different preparations shown in brackets.

Table 6.2. Release of Catecholamines by Cultured Chromaffin Cells.

| | Cell | | Medium | | |
|---|---|---|---|---|---|
| | Adrenaline ($nmol/10^6 Cells$) | Noradrenaline ($nmol/10^6 Cells$) | Adrenaline ($nmol/10^6 Cells$) | Noradrenaline ($nmol/10^6 Cells$) | Release. (%) |
| Control. | 119 | 29 | 1.4 | 2.2 | 2.4 |
| Potassium (50mM). | 125 | 28 | 5.4 | 5.6 | 6.7 |
| Nicotine (10uM). | 120 | 30 | 19.0 | 10.4 | 16.4 |
| Nicotine + Tubocurarine (100uM). | 142 | 36 | 1.6 | 2.4 | 2.2 |

The release of catecholamines into the cell medium is expressed as a percentage of the total catecholamine content (cellular plus medium).

stained with acridine orange and ethidium bromide (AO/EB) for fluorescence microscopy. This treatment gave slightly higher estimates of cell viability (98%) than trypan blue exclusion and in addition a qualitative impression of the health of cell preparations from the strength of their fluorescence signal. Figure 6.1b shows cells (20μm in diam.) fluorescing bright green, with non-viable cell nuclei staining with ethidium bromide.

Figure 6.1c&d. show respectively a high power phase-contrast micrograph and fluorograph of freshly isolated chromaffin cells after purification on a Percoll gradient. The cells are again phase bright with large nuclei and a granular cytoplasm. One or two non viable cells (arrowed) have a crinkley swollen appearance and stain with EB. The dye neutral red has been reported to specifically stain chromaffin cells (Role and Perlman, 1980; Wilson and Viveros, 1981) and like AO it partitions into lysosomes and secretory granules. However, in this study no convincing differences between the cell types present in these prepartions could be shown with this dye.

Figure 6.2 shows chromaffin cells in culture after (a) 18hr and (b) and (c) 3-4days. After overnight culture the cells have only just begun to spread on the plastic surface and in many preparations cells aggregated into strings and this appeared to hinder attachment and spreading. Providing cells were not seeded at high density they produced axon-like processes (neurites) after a few days in culture; characteristic of chromaffin cells cultured in serum containing media (Figure 6.2b). At high plating densities contact inhibition prevented the production neurites (Figure 6.2c). Cell attachment and spreading was markedly improved if culture dishes were coated with rat tail collagen (not shown).

Percoll Fractionation.

Chromaffin cells can be fractionated by isopycnic centrifugation in Percoll, equilibrating at a greater density than many other cells present in medullary preparations. The top part of the gradient containing cell debris, cortical cells and other cell types was discarded. Identification of cortical cells by their morphology was not possible, however they have been unequivocally identified by other workers by their large content of mitochondria in electron micrographs. Chromaffin cells can be characterised biochemically by their high catecholamine levels. Chromaffin cell fractions enriched in total catecholamines and with increased adrenaline to noradrenaline ratios were recovered from the Percoll gradient (Table 6.1). Below a flocculent band of debris at the top of the gradients was a broad band of cortical cells (density 1.054g/ml), a broad band of chromaffin cells mainly adrenaline secreting, and finally a compact red band of erythrocytes (density 1.130g/ml) near the bottom of the tube.

Chromaffin cells have a density of 1.060 g/ml in Percoll (Livett et al., 1979). Using density marker beads (from Pharmacia) chromaffin cells (density 1.067g/ml) were collected between densities of 1.060 and 1.081g/ml. Purified chromaffin cells had adrenaline to noradrenaline ratios which were 3-fold enriched over the crude cell isolate (see Table 6.1).

Catecholamine Release.

The catecholamine content of cultured chromaffin cells (shown in Figure 6.2) was monitored and the pharmacological effects of high potassium, nicotine, and tubocurarine were examined (see Table 6.2). After 48hr in the presence, and 24hr in the absence of serum the

cells had maintained their catecholamine content. Both high potassium (50mM) and nicotine (10μM) stimulated release of catecholamines; a characteristic of cultured chromaffin cells. Release was blocked by a 30min preincubation with the antagonist tubocurarine (100μM).

**Differential Plating of Cells.**

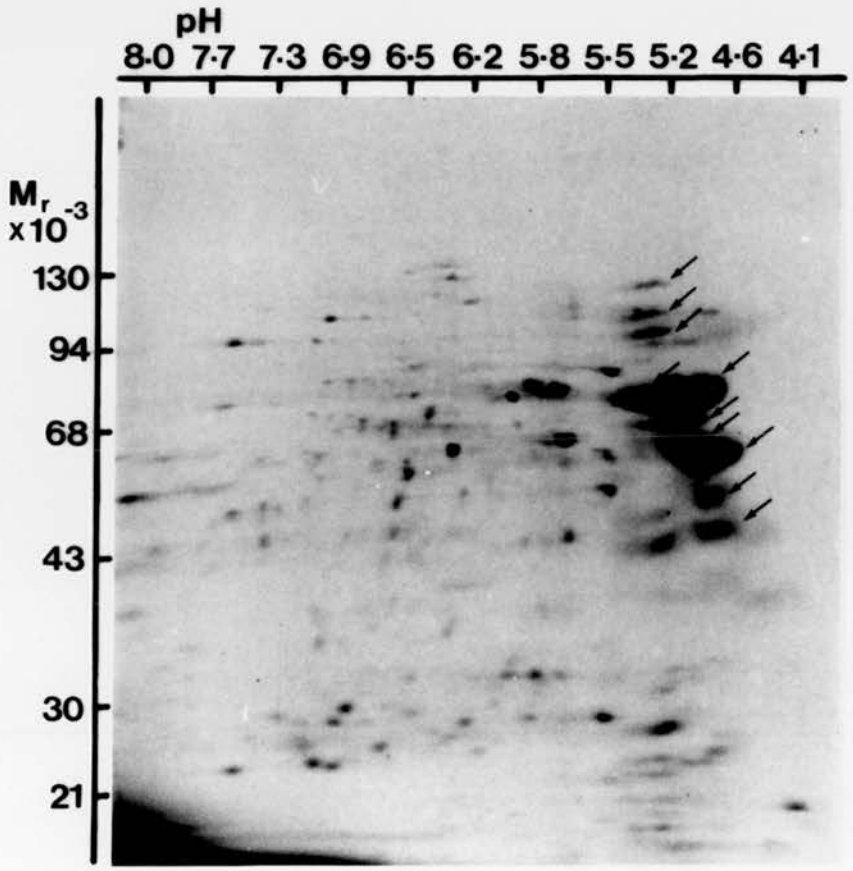
Chromaffin cells take much longer to adhere and spread on plastic than many of the other cell types. This was used to advantage for the further purification of cells before use in labelling experiments. By incubating cells in serum free medium in a $75cm^2$ plastic culture flask for an hour at $37^oC$ chromaffin cells were recovered by gentle shake-off while other cell types remained firmly adhered to the flask.

**Cellular Synthesis of the Chromogranins.**

Figure 6.3 shows a two-dimensional electrophoretogram of whole bovine adrenal medullary cell proteins stained with Coomassie Blue. The acid region of the polypeptide map is dominated by chromogranin A and its proteolytic breakdown products and is almost identical to the map produced by chromaffin granule lysate (see Figure 4.4). The chromogranin families are indicated by arrows in Figure 6.3 and have been identified by immune blotting (Fischer-Colbrie and Frishenschlager, 1985). That chromogranin A dominates this map is not surprising since it may account for up to 7% of the total medullary cell protein (O'Connor and Frigon, 1984).

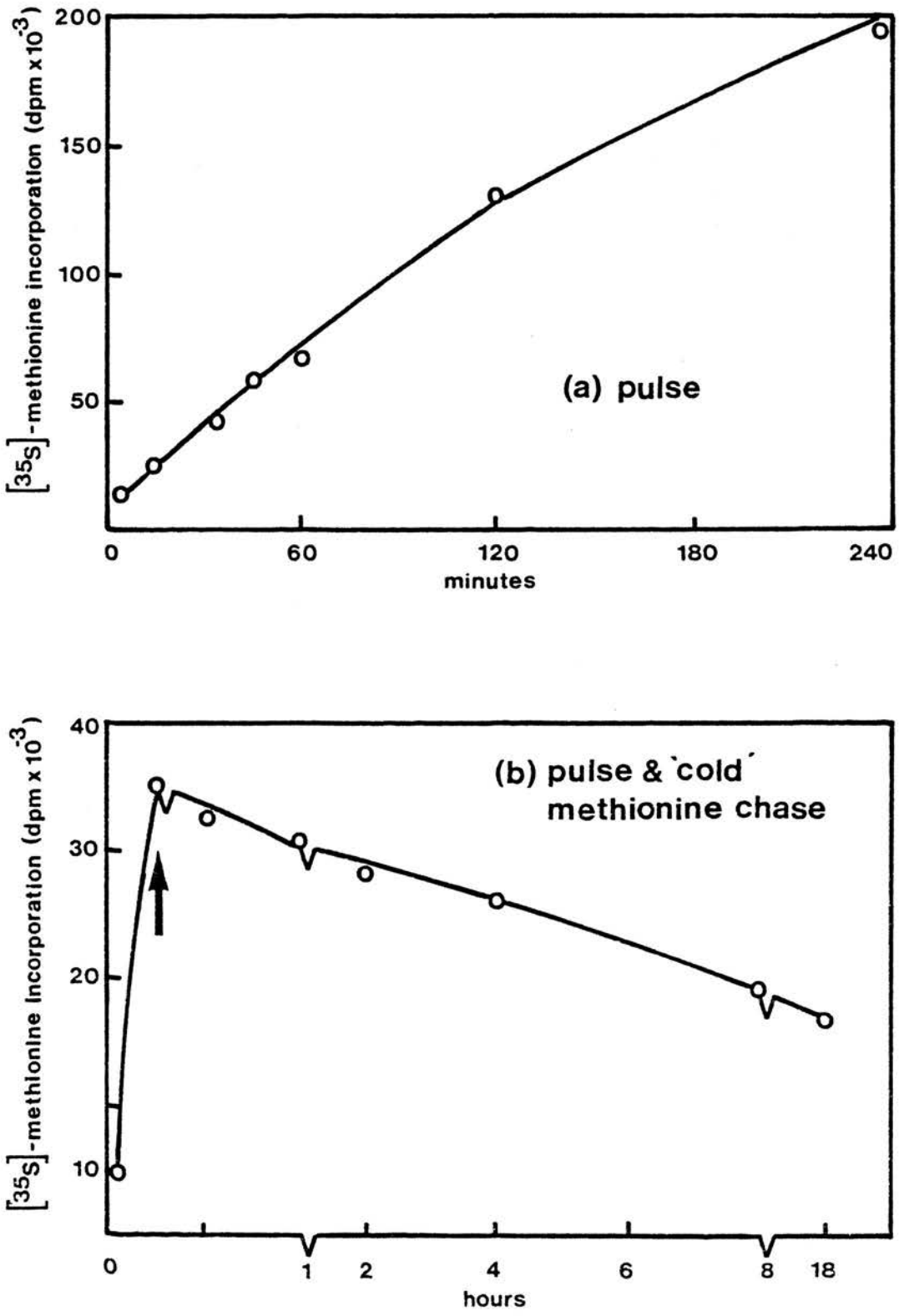
To follow the cellular synthesis of the chromogranins freshly isolated cells were labelled with [$^{35}$S]-methionine. Figure 6.4a shows the incorporation of [$^{35}$S]-methionine into newly

Figure 6.3. Two-Dimensional Electrophoretic Analysis of Chromaffin Cell Proteins.




Chromaffin cells were isolated by collagenase digestion af adrenal glands and purified on a gradient of Percoll. The two-dimensional electrophoretogram of total chromaffin cell proteins (300µg) was stained with Coomassie blue. The major proteins of the chromaffin granule matrix (chromogranins) are arrowed.

Figure 6.4. Incorporation of [$^{35}$S]-Methionine into Newly Synthesised Chromaffin Cell Proteins.



synthesised chromaffin cell proteins. Under these conditions incorporation was linear for at least 2hr. The addition of cold methionine (10mM) (Figure 6.4b) after an initial pulse for 15min was effective in reducing the specific activity of the label to enable the kinetics of post-translational processing to be monitored.

Initial attempts to immunoprecipitate radiolabelled chromogranins from cell lysates were unsuccessful because of the high content of endogenous protein. Electrophoretograms were therefore stained with Coomassie blue before fluorography, dried, then aligned with autoradiographs by using spots of radioactive ink. Many proteins could be identified by their characteristic shape, molecular mass and isoelectric point. Three spots were identified as chromaffin granule secretory proteins. Apart from the chromogranins, about five relatively major proteins which did not appear to be chromaffin granule proteins were synthesised.

**1. Chromogranin A**

The most distinctive spot on the fluorograms is chromogranin A (Figure 6.5b). This polypeptide shows relative molecular mass and isoelectric point heterogeneity that is characteristic of the mature protein shown in Figure 4.4. Such heterogeneity is shown by many glycosylated proteins, and the mature form of chromogranin A is O-glycosylated. The kinetics of glycosylation can be deduced from pulses with radiolabel as shown in Figure 6.5. After 10min there is no sign of the mature glycosylated form of chromogranin A ($M_r$ 70,000; pI4.8) seen in Figure 6.5b after a 60min pulse flaring out from a more basic polypeptide of lower molecular mass (pI 5.2; $M_r$ 68,000). The unglycosylated polypeptide is identical to the polypeptide synthesised by translation of adrenal medullary mRNA <u>in vitro</u> in the presence of dog pancreas

**Figure 6.5. Two-Dimensional Fluorograms of [$^{35}$S]-Methionine Labelled Chromaffin Cell Proteins.**

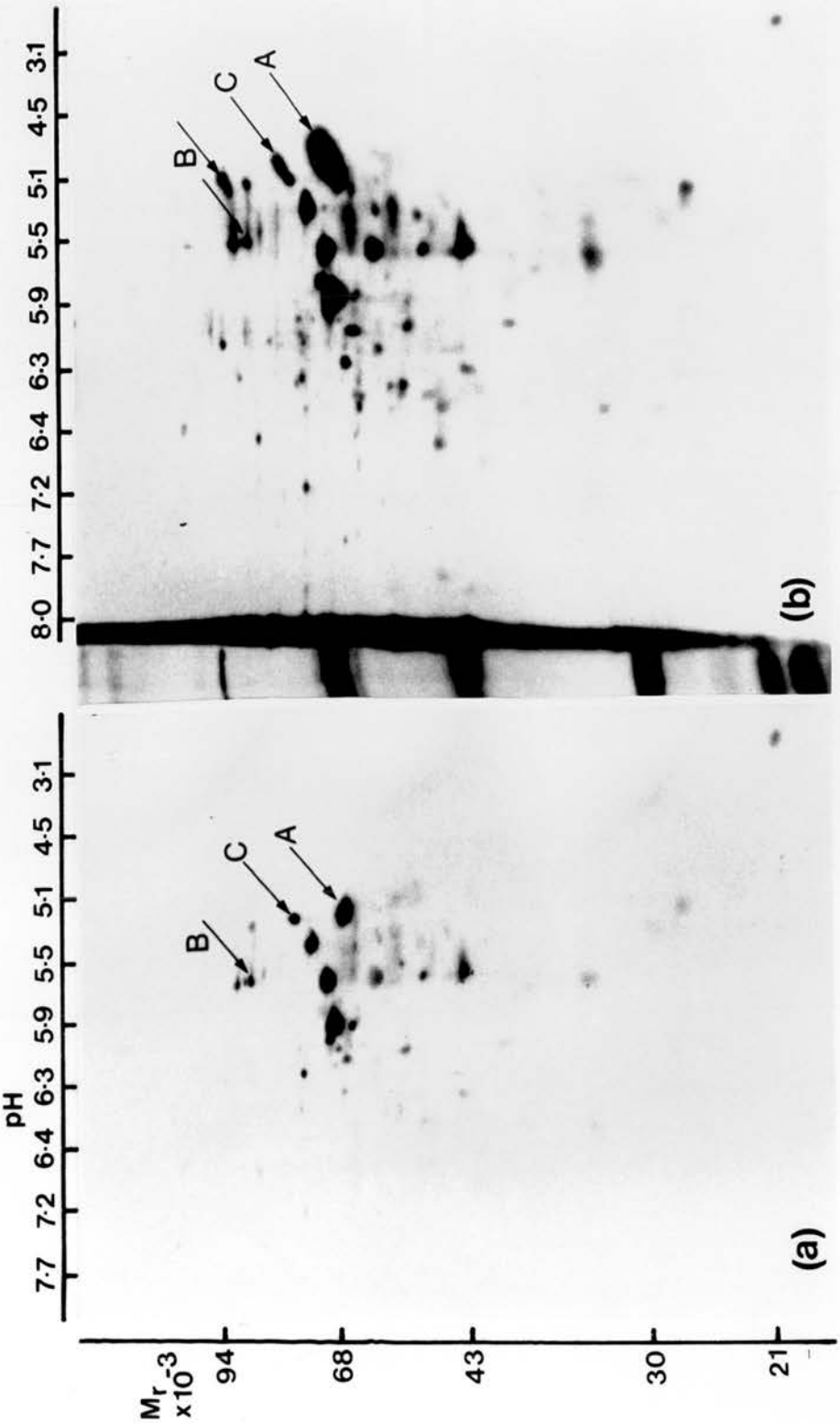
Freshly isolated chromaffin cells ($2.5 \times 10^6$ cells/ml) were labelled with [$^{35}$S]-methionine (50$\mu$Ci/ml) for (a) 10min and (b) 60min. (These cells were not purified on Percoll gradients).

After incubation, cells were chilled, sedimented and proteins solubilised and separated by two-dimensional electrophoresis using a 7-15% gel in the second dimension. The gels were stained with Coomassie blue before processing for fluorography.

In (a) the three unglycosylated precursor polypeptides of the chromogranin families of proteins are indicated by arrows: chromogranin A, A; chromogranin B, B; chromogranin C, C.

In (b) the glycosylated chromogranins show IEF shifts to the acidic end of the gel with a concomitant $M_r$ shift.

Figure 6.5.


pH
7·7 7·2 6·4 6·3 5·9 5·5 5·1 4·5 3·1
8·0 7·7 7·2 6·4 6·3 5·9 5·5 5·1 4·5 3·1
$M_r$ x10$^{-3}$
94
68
43
30
21
A
B
C
(a)
(b)

microsomes (Falkensammer et al., 1985; Kilpatrick, 1985). However, pulse and chase experiments, an example of which is shown in Figure 6.6, show that glycosylation of the protein can just be detected around 20min after the initial pulse of label (not shown) and that after a 60min chase chromogranin A is fully glycosylated and shows no sign of further post-translational modification. However, immunoblotting analysis of tissue fractions shows that it does undergo proteolysis (shown also in Figure 6.3), presumably in mature secretory granules. When isolated cells were labelled for 4,8,12 and 18hrs they showed no sign of any low $M_r$ products of proteolysis.

2. Chromogranin B.

The Chromogranin B family of polypeptides (Fischer-Colbrie and Frischenschlager, 1985; Falkensammer et al., 1985b) are more basic than the chromogranin A family (see Figure 6.5b). The parent protein has an $M_r$ of 100,000 and pI5.2. Following a labelling pulse for 10min with [$^{35}$S]-methionine there is no polypeptide spot visible on fluorographs (Figure 6.6a) in the position corresponding to that of chromogranin B. However after a 50min chase it can be identified (Figure 6.6b) and there is concomitant disappearence of a polypeptide with $M_r$ of 96,000 and pI5.8 (arrowed in Figure 6.6a). In marked contrast to the chromogranin A precursor this polypeptide shows a large shift in isoelectric point, suggestive of a major post-translational addition of acidic residues. This precursor polypeptide appears to be identical to the newly synthesised product which has been immunoprecipitated with an antiserum to the mature protein from in vitro translation systems in the presence of microsomal fractions from dog pancreas and shown to incorporate radioactive sulphate (Falkensammer et al., 1985b)

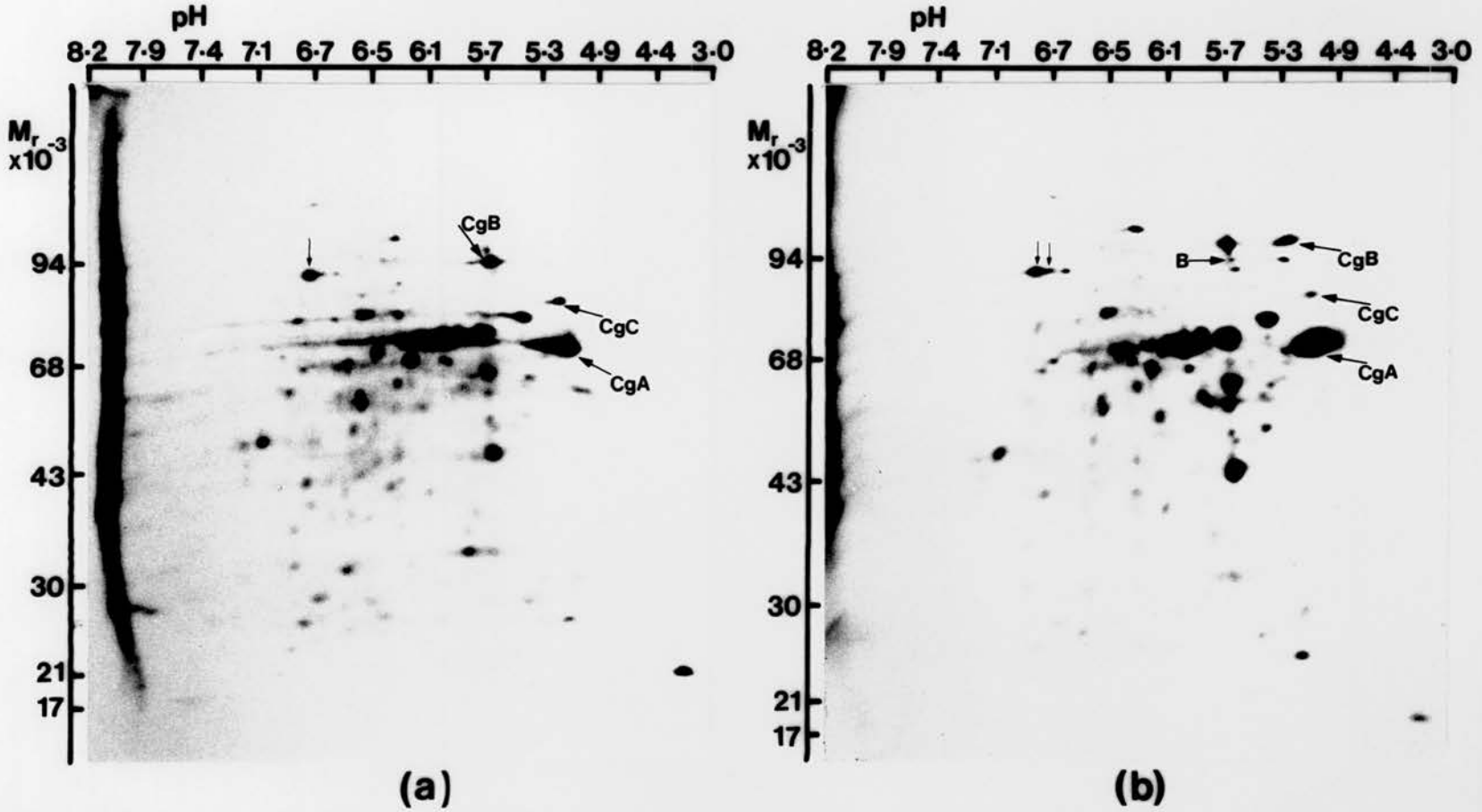
Figure 6.6. Two-Dimensional Fluorograms of Chromaffin Cell Proteins Labelled With [$^{35}$S]-Methionine: Pulse and Chase.

In this experiment freshly isolated and Percoll purified chromaffin cells ($2 \times 10^6$ cells/ml) were labelled with [$^{35}$S]-methionine (50$\mu$Ci/ml) for (a) 10min, then chased for 50min by the addition of cold methionine (b).

In (a) the unglycosylated precursor polypeptides of the chromogranin families of proteins are shown: chromogranin A, CgA; chromogranin B, CgB; chromogranin C, CgC. The unlabelled arrow indicates an $M_r$ 90,000 polypeptide which is the only other one to undergo any IEF shift, to the basic end of the gel, during the chase period [see double arrows in (b)].

In (b) the glycosylated chromogranins show IEF shifts to the acidic end of the gel with concomitant disappearance of the precursor polypeptides during the chase period; a small amount of CgB precursor (B) is indicated.

**Figure 6.6.**


pH
8·2 7·9 7·4 7·1 6·7 6·5 6·1 5·7 5·3 4·9 4·4 3·0
$M_r$ x$10^{-3}$
94
68
43
30
21
17
CgB
CgC
CgA
(a)
pH
8·2 7·9 7·4 7·1 6·7 6·5 6·1 5·7 5·3 4·9 4·4 3·0
$M_r$ x$10^{-3}$
94
68
43
30
21
17
B
CgB
CgC
CgA
(b)

3. Chromogranin C.

Figures 6.5b and 6.6b show that only one other polypeptide undergoes an acidic shift in IE point and an increase in $M_r$. It has $M_r$ 84,000 and pI5.2 and this previously unidentified polypeptide behaved like chromogranin A, in that it was post-translationally modified, apparently by glycosylation. The mature form of this protein ($M_r$86,000, pI5) can be identified as a major Coomassie staining spot on electrophoretograms of chromaffin granule lysates (Figure 4.4). This protein has recently been isolated and shown to have immunological identity with Secretogranin II from anterior pituitary (Fischer-Colbrie et al., 1986).

4. Other Proteins.

Only one other polypeptide appears to undergo a shift in isoelectric point, moving to the basic end during focusing (Figures 6.5 and 6.6). Its $M_r$ is about 150,000 and it shifts about 0.5 pH units. It does not appear to be a chromaffin granule protein, as shown by its exclusion from microsomes (discussed below).

Separation of Secretory and Cytoplasmic Proteins.

Secretory proteins are synthesised on bound polysomes and are translocated across the membrane of the RER into its lumen. These proteins can therefore be separated from the pool of cytoplasmic proteins by recovery of a microsomal fraction by high speed centrifugation. In order to identify such proteins, microsomal fractions were prepared (as described in Chapter 2, page 80) from both cells pulse-labelled for 10min with [$^{35}$S]-methionine and from cells labelled and chased for 50min (as shown in Figure 6.6) with cold methionine. These fractions were then incubated with pronase in the presence or absence of Triton X-100.

Figure 6.7a shows a fluorogram of the microsomal fraction before incubation with pronase. There are eight major polypeptides, three of which(indicated by arrows) correspond to the post-translationally unmodified forms of the chromogranins as discussed above. Comparison of Figure 6.7a with Figure 6.6a, of whole cells labelled for 10min shows that the putative chromogranin A precursor is present in the microsomes as a doublet of spots; an extra polypeptide of $M_r$69,000 and pI 5.3 is present under the conditions used in this experiment (arrowed in Figure 6.7a), rather than the single polypeptide seen previously. This is clearly seen by comparing the chromogranin A spot on the fluorogram with the chromogranin C spot above it, and comparing this pattern with that in Figure 6.7b. The putative chromogranin C precursor ($M_r$84,000, pI5.2) together with the putative chromogranin B precursor are also clearly segregated into the microsomes, and treatment of the microsomes with pronase confirms that they are secretory proteins.

Figure 6.7b shows that when the microsomal fraction is subjected to pronase treatment, the chromogranin A doublet collapses into one spot, characteristically shaped like the spots seen in previous 10min pulses (Figure 6.5a and 6.6a). This is indicative of some of this protein being exposed on the cytoplasmic face of the microsomes, while, most is protected.

Other proteins are mostly unaffected by the pronase treatment. However, this appears to be because they are resistant to this protease and also to trypsin and chymotrypsin (not shown), since they remain after pronase treatment in the presence of Triton X-100. We therefore cannot say whether they are adherent cytosolic proteins or are within the lumen of the RER (Figure 6.7c).

Figure 6.7. Secretory Protein Precursors are Protected From Proteolysis Within Microsomal Membranes: Identification of Chromogranins.

This figure shows newly synthesised adrenal medullary microsomal proteins labelled after a 10min pulse with [$^{35}$S]-methionine. The cells were chilled, homogenised and microsomes isolated as described in Chapter 2 (page 80), then treated with: (a) control (no pronase); (b) pronase (0.2mg/ml; 0.1mMCaCl ); (c) pronase plus 1% Triton X-100 to permeabilise membranes.

After incubation on ice for 60min samples were precipitated with ice cold TCA before electrophoretic analysis. Each gel contains the microsomal fraction recovered from $20x10^6$ cells. The radiolabelled chromogranins were identified by aligning the fluorogram with the Coomassie blue stained and dried gel.

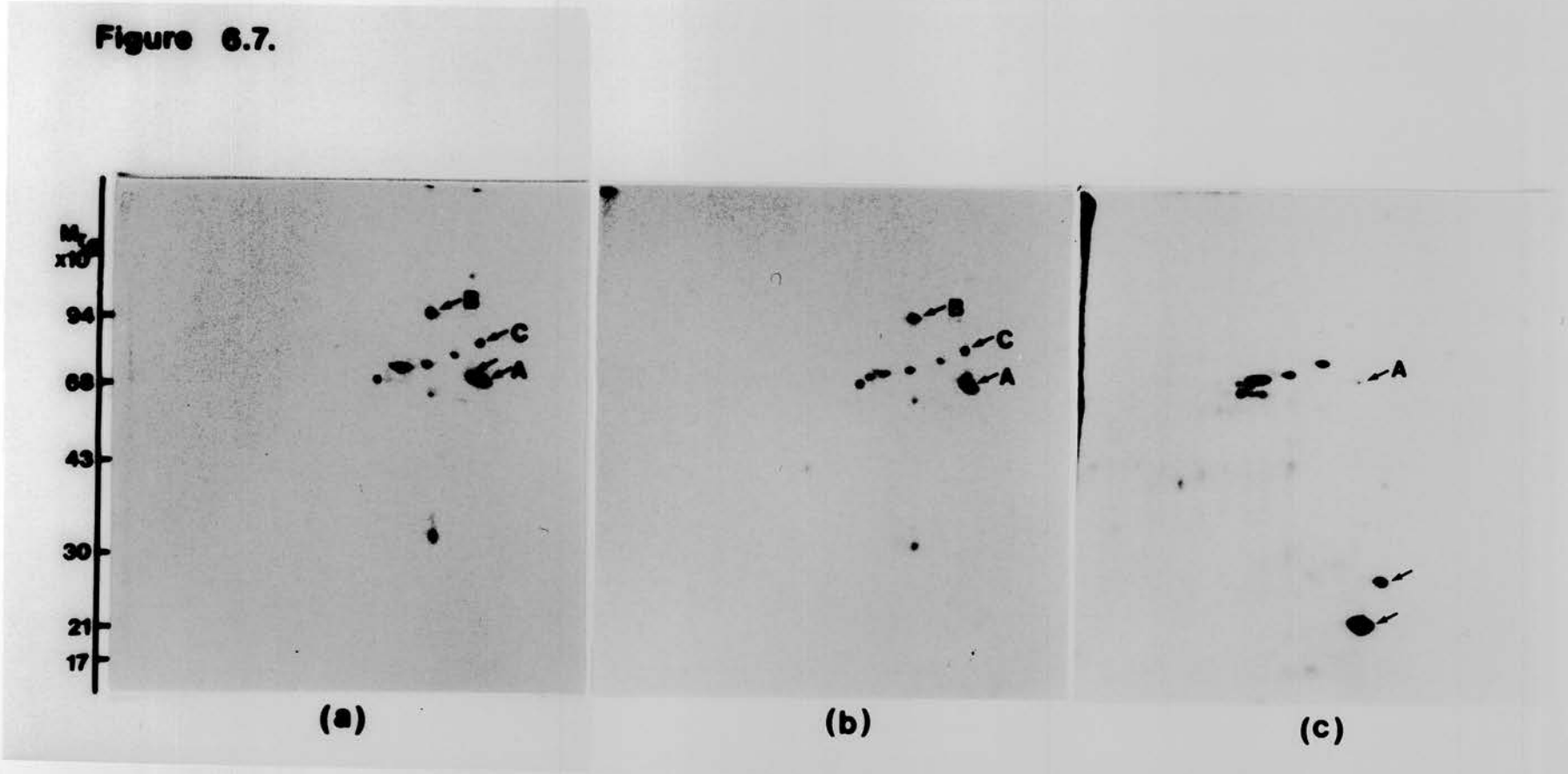
The arrows indicate: chromogranin A, A; chromogranin B, B; chromogranin C, C. The unlabelled arrow in 6.7a indicates an additional CgA polypeptide previously not identified. At shorter exposures the CgA spot is clearly a doublet. It still remains to be demonstrated that these are immunologically identical. However after treatment with pronase they migrate as a single spot which is apparently identical to the polypeptide produced in cell-free systems in the presence of microsomes.

The apparent shift of the polypeptide map to the basic end of the focusing gel is probably a consequence of the additional nonionic detergent present in this fraction.

Figure 6.8. Mature Secretory Proteins Are Protected From Pronase Within Membrane Vesicles.

This figure shows the Coomassie stained electrophoretograms of adrenal medullary microsomal fractions described in Figure 6.7 above.

**Figure 6.7.**



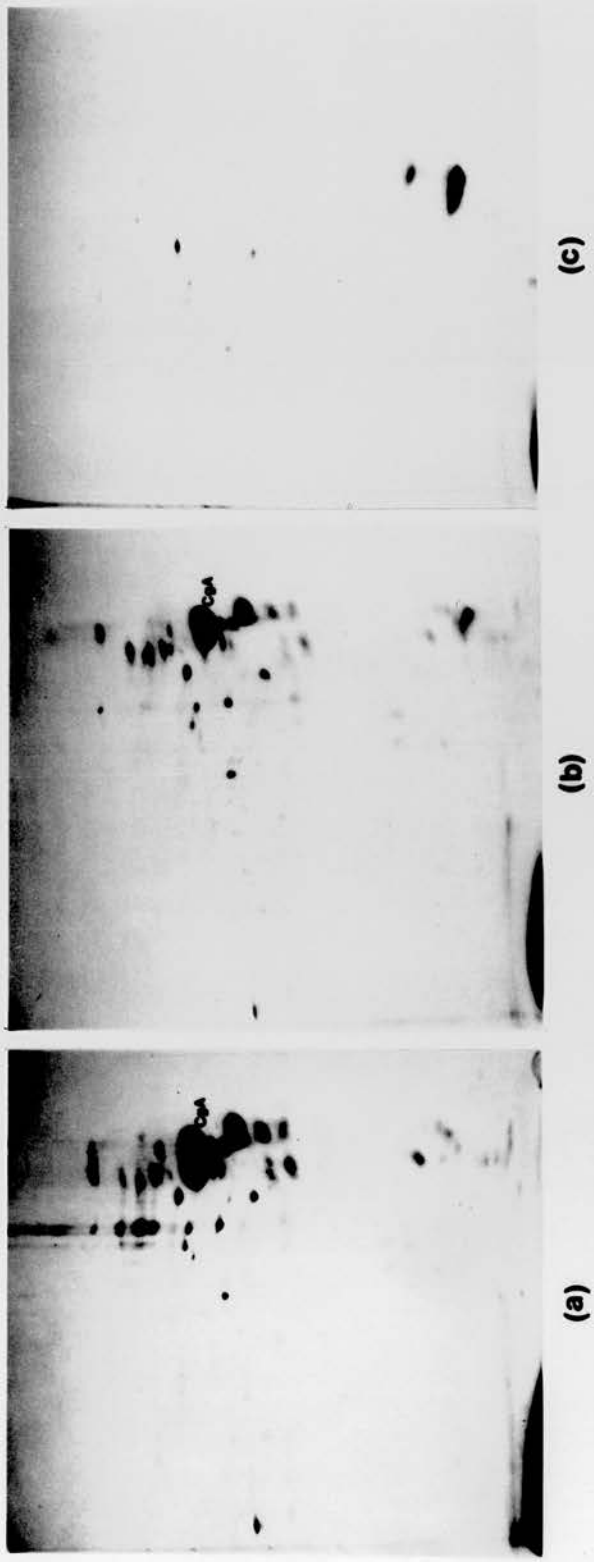
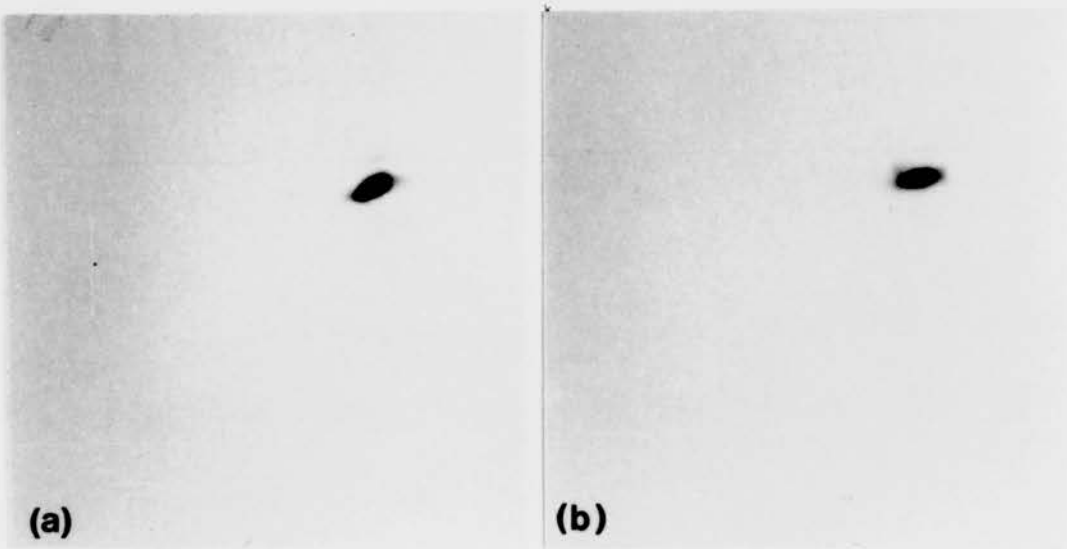



Figure 6.8.

All three chromogranin precursors are lost during pronase treatment in the presence of Triton, confirming that they were previously protected by membranes. However, two new spots of about $M_r$25,000 and 30,000 appear (arrowed in Figure 6.7c). Their high intensities suggest that they may be pronase-resistant products of chromogranin A digestion. The mature glycosylated proteins (shown in Coomassie stained gels in Figure 6.8a) like their biosynthetic precursors were also protected from proteolysis in microsomal fractions (Figure 6.8b) suggesting that Golgi membrane vesicles and immature chromaffin granules are isolated intact. In the presence of Triton X-100 the mature chromogranins were digested, suggesting that addition of O-linked carbohydrate does not protect them from this protease (Figure 6.8c). The pronase resistant chromogranin A fragments ($M_r$25&30,000) are more acidic than those of the unglycosylated precursor fragments (compare Figure 6.7c with 6.8c).

Unambiguous identification of chromogranin A requires immunoprecipitation. This has been unsuccessful in the hands of previous workers (Falkensammer et al., 1985) because of the endogenous protein in the cells. My initial attempts at immunoprecipitation of chromogranin A were also unsuccessful. However this protein was eventually immunoprecipitated from lysates from $5x10^6$cells labelled with methionine at 100$\mu$Ci/ml and immunoprecipitation carried out with fifty times the previous amount of antiserum. The precaution of adding fresh protease inhibitors at each stage of the procedure was taken. Figure 6.9 shows fluorograms of chromogranin A immunoprecipitated with antisera to the mature polypeptide after a 30min and a 2hr labelling period with [$^{35}$S]-methionine. The immunoprecipitated spot after 30min is begining to show signs of glycosylation, however in Figure 6.9a the

Figure 6.9. Cellular Synthesis and Immunoprecipitation of Chromogranin A.




Percoll purified chromaffin cells ($5x10^6$cells/ml) were preincubated in RPMI methionine free medium at $37^oC$ in an atmosphere of 95%$O_2$5% $CO_2$ before incubation in fresh medium containing [$^{35}S$]-methionine (100$\mu$Ci/ml).

(a) 30min pulse and (b) a 2hr pulse. Cells were chilled and recovered by centrifugation then whole cell lysates processed for immunoprecipitation as described in Chapter 2 (page 67). Before immunoprecipitation with antichromogranin A antiserum lysates were immunoprecipitated with anti-DBH antiserum.

spot intensity is more basic and has a lower $M_r$ than the fully glycosylated polypeptide immunoprecipitated after the 2hr labelling period (Figure 6.9b). However it is begining to show signs of its glycosylation. Ideally one should be able to show co-migration in two dimensions on electrophoretograms of cell-free product in the presence of microsomal fractions, with the products of cellular synthesis after pulse chase experiments. While this experiment was tried a good few times, getting enough label into chromogranin A during a 10min pulse was not successful under the conditions used above. Using more antiserum tended to lead to large amounts of IgG on focusing gels which distorted the focusing patterns.

**Inhibitors of Protein Biosynthesis.**

An approach that has been successful in dissecting the secretory pathway is to challenge cells with agents which cause blocks in protein biosynthesis at specific sites. This allows intermediates to either accumulate at the block or in other cases it prevents post-translational modifications, the absence of which may not necessarily impair transport.

1. FCCP.

The biosynthetic pathway for secretory protein processing is energy dependent (Harwood et al., 1976). Uncouplers, such as FCCP, of oxidative phosphorylation block transport from the RER to the Golgi complex without observable morphological changes; these inhibitors are thought to act at a pre-Golgi site (Tartakoff and Vassilli, 1977; Datema and Schwarz, 1981). They do not affect synthesis of the lipid-linked $Glc_3Man_9GlcNAc_2$ which is still transfered to N-glycosylated proteins - suggesting that there may be dolichol phosphate precursor pools (Datema and Schwarz, 1981).

Figure 6.10 shows fluorograms of proteins synthesised by cells in the presence of 10μM-FCCP. After a 15min pulse of methionine (Figure 6.10a) the pattern is identical to a control pulse in the absence of this inhibitor (see Figure 6.5a and 6.6a). However cellular protein synthesis is about five times less efficient in the presence of FCCP, presumably a consequence of the general depletion of cytoplasmic ATP levels. FCCP at a concentration of 1uM inhibited protein synthesis by about two-fold.

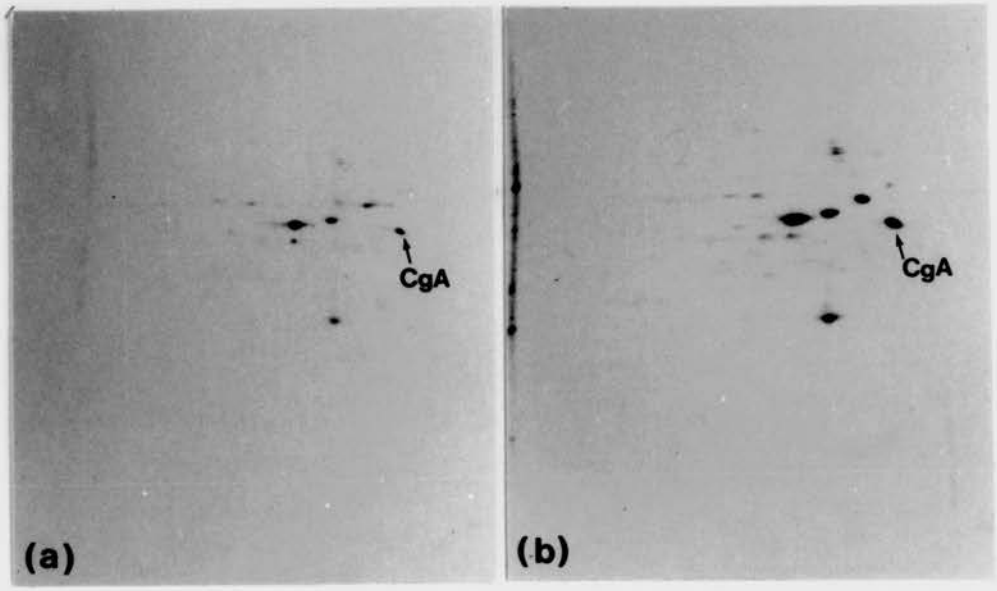
After a 45min chase (Figure 6.10b) there is no sign of chromogranin glycosylation (compare with Figure 6.5b and 6.6b), suggesting that at these low concentrations, FCCP is inhibiting the transport of newly synthesised secretory protein to the Golgi complex. Thus transport of proteins from the RER to the Golgi complex is exquisitively sensitive to changes in cytosolic ATP levels. Of relevance to the effects of monensin (see below), is the fact that the chromogranin A precursor does not appear to be affected by any proteolytic breakdown within the compartment distal to the Golgi complex in which it is trapped.

2. Monensin.

Monensin, a $Na^+/H^+$ ionophore, has been used to disrupt the transport of secretory proteins from the Golgi complex. Figure 6.11 shows fluorograms of protein synthesis in the presence of monensin. Unlike FCCP this inhibitor appears to have little effect on the level of protein synthesis. However the striking feature of these electrophoretograms is the absence of all three families of the chromogranins in both the pulsed and chased cells. The normal positions of the biosynthetic precursors in control cells, is marked by arrows in Figure 6.11a).

Why the chromogranins have disappeared after monensin

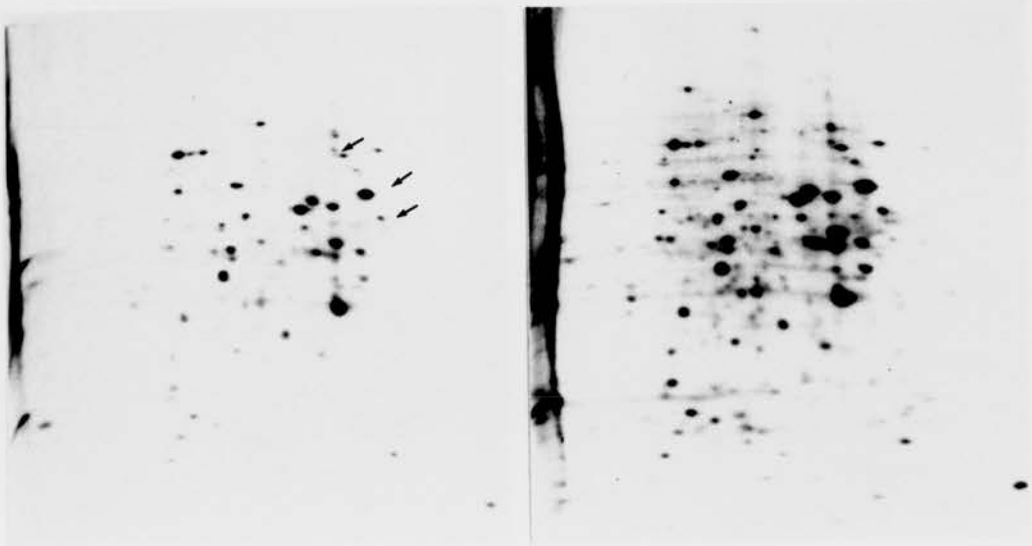
Figure 6.10. The Effect of FCCP on the Cellular Synthesis of the Chromogranins.




Chromaffin cells ($2x10^6$/ml) were incubated in methionine deficient RPMI medium containing 10μM FCCP for 30min at 37°C in an atmosphere of 95% $O_2$ 5% $CO_2$. The cells were then recovered by centrifugation at 1000rpm for 10min and resuspended in fresh medium containing 50μCi/ml [$^{35}$S]-methionine. The two-dimensional fluorograms show: (a) 15min pulse in the presence of 10μM FCCP; (b) a 45min chase with cold methionine in the presence of 10μM FCCP.

The precursor chromogranin A (CgA) polypeptide is indicated. Note that chromogranin A fails to become glycosylated after the 45min chase.

Figure 6.11. The Effect of Monensin on the Cellular Synthesis of the Chromogranins.



(a) (b)

Chromaffin cells ($2.5 \times 10^6$/ml) were incubated in maintainence medium containing 0.1-10μM monensin for 3hr at 37°C in an atmosphere of 95% $O_2$ 5% $CO_2$. The cells were then recovered by centrifugation at 1000rpm for 10min and resuspended in RPMI methionine deficient medium containing monensin. After a further incubation for 60min the cells were again recovered by centrifugation and resuspended in the same fresh medium containing 50μCi/ml [$^{35}$S]-methionine. The two-dimensional fluorograms show: (a) 15min pulse in the presence of 10μM monensin; (b) a 45min chase with cold methionine in the presence of 10μM monensin.

The arrows indicate the positions in which the chromogranin precursor polypeptides are normally found in the control gels (see Figure 6.6)

treatment will be discussed later. This result does however provide us with some indirect information about the chromogranins. The monensin effect shows that there is a biosynthetic relationship between the polypeptide ($M_r$84,000, pI 5.2) called chromogranin C and the other two chromogranin precursors. In addition, as is shown in Figure 6.5a, the area around the chromogranin B precursor is crowded with polypeptides and in the absence of immunoprecipitation data in this present work, this result allows the indirect confirmation of the identities of the polypeptides which represent the precursor and mature chromogranin B spots.

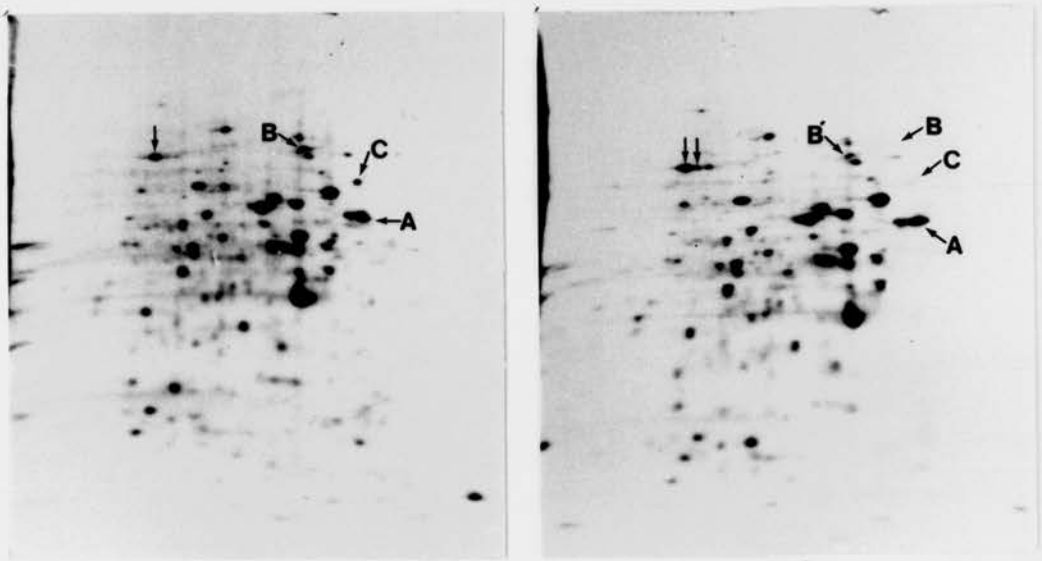
Monensin causes the single $M_r$150,000 polypeptide which shifts to the basic end of the isoelectric focusing gels (indicated in Figure 6.6 and Figure 6.12) to be synthesised and migrate as the mature polypeptide after a 15min pulse in the presence of this drug.

3. Tunicamycin.

Tunicamycin inhibits the attachment of high mannose core oligosaccharides to proteins. Chromogranin A, having only O-linked oligosaccharides, should not be affected by this inhibitor. Labelling of cells with [$^{35}$S]methionine was therefore carried out in the presence of tunicamycin to see if any changes in the post-translational modifications identified by two-dimensional electrophoresis occurred in the presence of this inhibitor.

Figure 6.12 shows fluorograms of proteins synthesised by cells preincubated for 4hr with 10µg/ml of tunicamycin. These two fluorograms should be compared with those shown in Figure 6.6. Following a 10min pulse with [$^{35}$S]-methionine all three of the chromogranins were identified (Figure 6.12a) and were identical to control incubations in the absence of the inhibitor. After a 50min chase with cold methionine in contrast to the controls (see

Figure 6.12. The Effect of Tunicamycin on the Cellular Synthesis of the Chromogranins.




Chromaffin cells ($2.5 \times 10^6$; Percoll purified) were incubated in maintainence medium (50% DMEM/F12) containing 10µg/ml tunicamycin for 3hr at $37^{o}C$ in an atmosphere of $95\%O_2 5\%CO_2$. The cells were recovered by centrifugation, resuspended and incubated in methionine free RPMI medium containing tunicamycin, for 60min. Cells were then labelled in fresh medium containing 50µCi/ml [$^{35}$S]-methionine and 10µg/ml tunicamycin.

Fluorogram (a) shows a 10min pulse in which the chromogranin precursors are indicated: chromogranin A, A; chromogranin B, B; chromogranin C, C. The unmarked arrow indicates the $M_r$ 90,000 polypeptide (non-chromaffin granule). This fluorogram is similar to that shown in Figure 6.6a.

Fluorogram (b) shows the positions of the chromogranins [indicated as in (a)] after a 50min chase period. The chromogranin B precursor (B') appears to be more prominant in control gels (compare with Figure 6.6b) and only a small amount of mature CgB is evident.

Control gels run in the presence of the carrier solvent were identical to those shown in Figure 6.6.

Figure 6.6b) very little chromogranin B had been fully glycosylated. This is consistent with data from carbohydrate analyses which show the presence of N-linked sugars in purified chromogranin B (Fischer-Colbrie et al., 1982). Both chromogranins A&C appear to undergo normal glycosylation, shown by their shifts in $M_r$ and isoelectric point, however there was some variation in the spot size and shape over a number of experiments suggesting that glycosylation of these polypeptides may not be totally unaffected by this inhibitor under the conditions used in these experiments. One chromaffin granule glycoprotein with N-linked oligosaccharide which is likely to be affected by tunicamycin is DBH; however this protein cannot be identified on these fluorograms.

Dopamine $\beta$-hydroxylase.

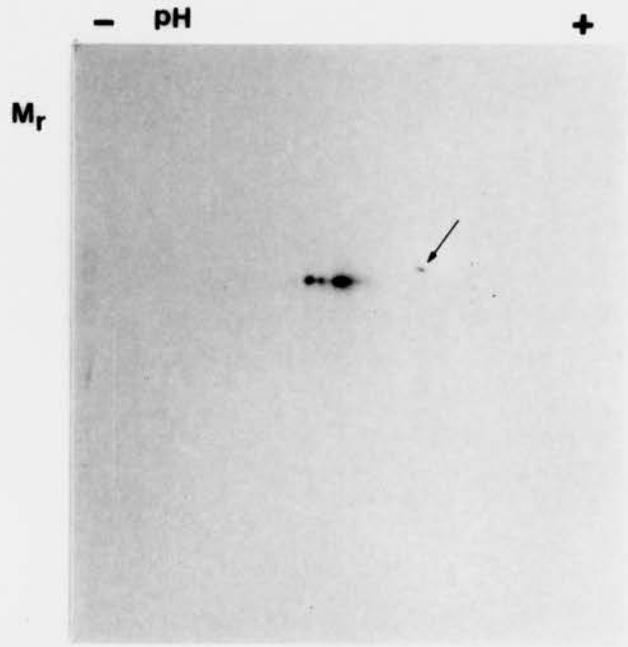
Immunoprecipitated sDBH proved difficult to resolve by two-dimensional gel analysis. However treatment of samples before IEF with SDS, an approach used successfully by Corcoran et al. (1982) to focus purified sDBH, produced a well resolved focusing pattern. Figure 6.13a shows a two-dimensional fluorogram of the polypeptides immunoprecipitated from a cell-free translation of adrenomedullary mRNA in the presence of dog pancreas microsomes (the translated proteins for immunoprecipitation were kindly supplied by L. Kilpatrick), identified a precursor which focused as a major spot of $M_r$67,000 and pI6 along with two minor companion spots which were slightly more basic. This heterogeneity in isoelectric point with no shift in molecular mass may be indicative of a focusing artifact. In the absence of microsomal membranes the major translation product is one of 46,000 (shown schematically in Figure 6.13b). A minor high $M_r$ species ($M_r$72,000) is present and the absence of the

**Figure 6.13. Immunoprecipitation of sDBH From Cellular and Cell-Free translation Lysates.**

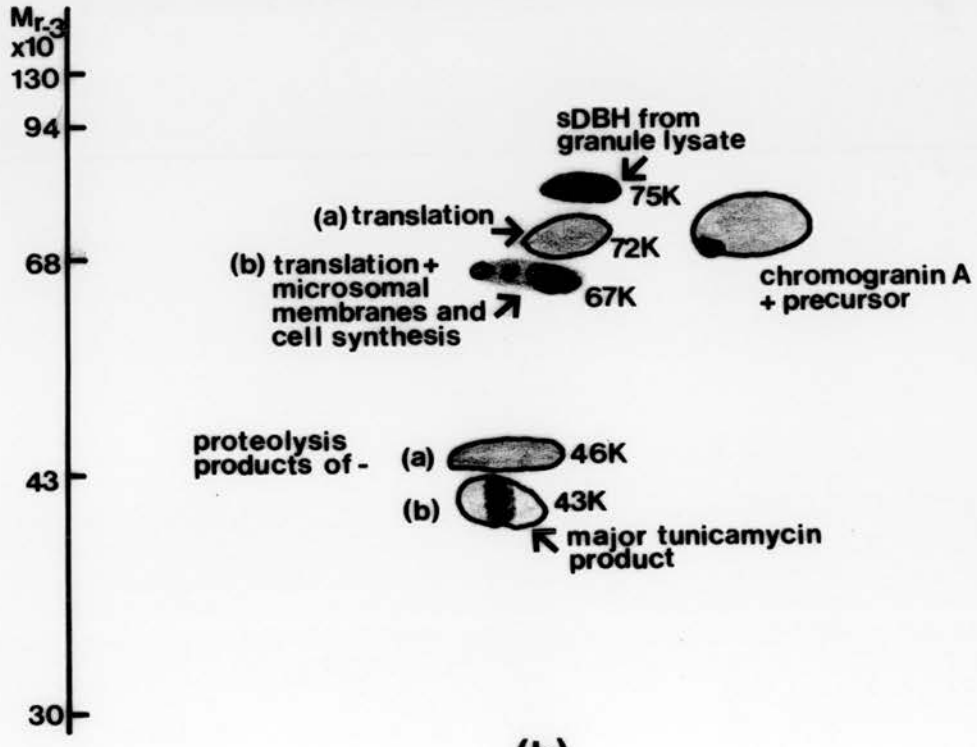
(a) shows a two-dimensional electrophoretogram of sDBH immunoprecipitated from a reticulocyte translation system in the presence of dog pancreas microsomes. Before isoelectric focusing the sample was solubilised in SDS. The arrow indicates precursor CgA which co-precipitated with this antiserum.

(b) This figure summarises the immunoprecipitation data obtained from both cell-free translation experiments (Kilpatrick, 1985) and cellular synthesis studies. The position of chromogranin A and its precursor are given as pI reference points. This diagram was composed by overlaying and tracing fluorograms and their respective Coomassie stained gels.

**Figure 6.13.**




(a)




(b)

$M_r$ 46,000 species in the presence of microsomes strongly suggests that the primary translation product is very susceptible to proteolysis. Figure 6.13b, which is a composite tracing from a number of fluorograms and the Coomassie blue stained and dried gels, shows that during maturation sDBH undergoes a marked shift in molecular mass with relatively little change in pI. This may not be surprising since in since in relation to GpIII for example it has a relatively small complement of sialic acid.

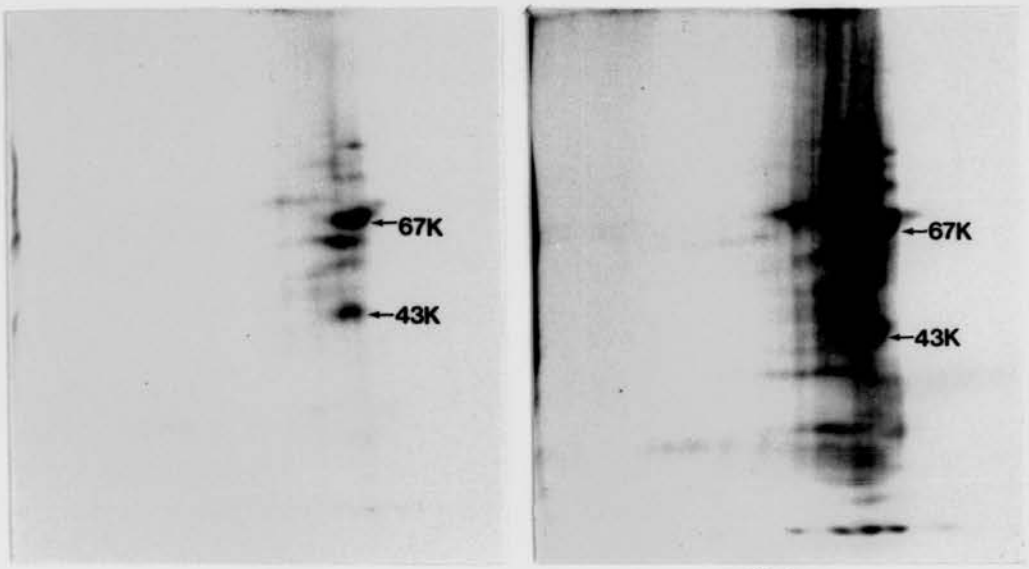
The DBH antiserum used in this experiment precipitated a small amount of the unglycosylated chromogranin A precursor, which is made abundantly in this system. This has provided an internal pI and $M_r$ standard (Figure 6.13a) with which to align the gels and fluorograms. This co-precipitation did not occur unless samples were treated with SDS prior to focusing.

**Cellular Synthesis of sDBH.**

Chromaffin cell proteins were labelled with [$^{35}$S]methionine, solubilised in detergent then immunoprecipitated with antisera to the mature sDBH (Figure 6.14). The immunoprecipitates were surprisingly complex, apparently due to extensive proteolysis and additionally complicated by a diffuse background of radiolabel. Preimmune serum did not precipitate any labelled proteins and immunoprecipitation in the presence of an excess of soluble DBH precipitated only a small amount of labelled protein.

Figure 6.14a shows a two-dimensional fluorogram of proteins immunoprecipitated from cells after 30min of labelling. The major product was a polypeptide of $M_r$ 67,000 apparently identical to the cell-free product translated in the presence of microsomes (Figure 6.13a). This sample was not treated with SDS before

Figure 6.14. Two-Dimensional Fluorogram of Dopamine $\beta$-Hydroxylase Immunoprecipitates.




Percoll purified chromaffin cells were labelled with [$^{35}$S]-methionine as described in the figure legend for 6.9.

Fluorogram (a) shows the proteins immunoprecipitated from whole cell lysates (described in Chapter 2; page 67) with antiDBH antiserum after labelling for 30min.

Fluorogram (b) shows labelling for 2hr and immunoprecipitation.

The two major polypeptides with counterparts in cell-free translation systems are indicated: 67K polypeptide and 43K polypeptide. The identity of the other polypeptides is uncertain, however their immunoprecipitation can be reduced in the presence of excess cold sDBH.

Microsomal fractions were also produced and proetins immunoprecipitated with antiDBH antiserum. These produced identical patterns to those shown here suggesting that all the major proteins precipitated are probably segregated within microsomal membrane compartments and not likely to be of cytoplasmic origin.

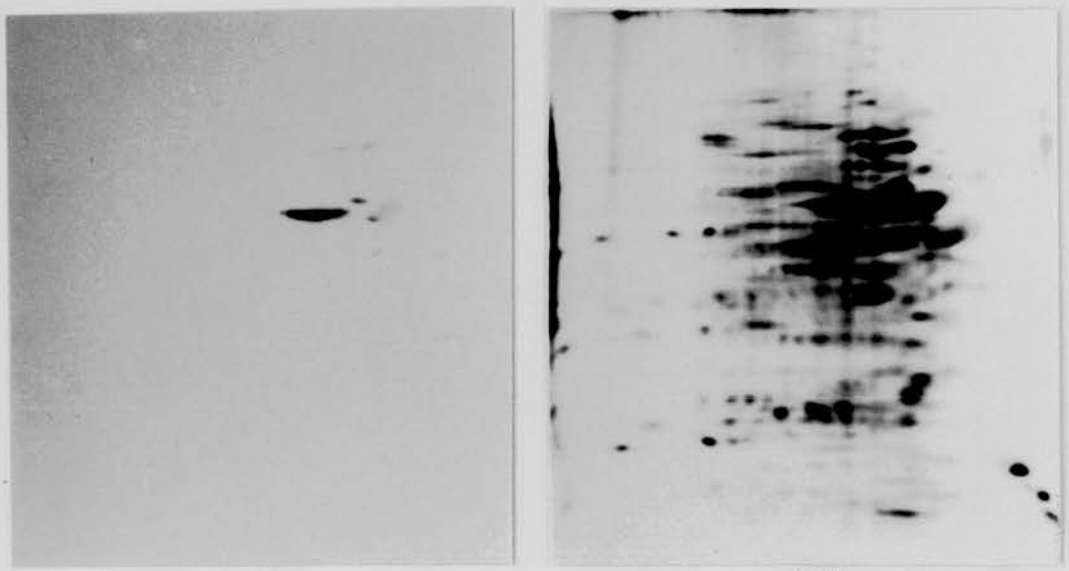
focusing. After extended periods of labelling (Figure 6.14b) a protein of $M_r$43,000 was predominant (Figure 6.14b) again suggesting proteolytic breakdown. This protein was not identical to the proteolytic product of the primary translation ($M_r$46,000) suggesting that the latter still contains signal sequence. In the presence of tunicamycin the only significant product immunoprecipitated was a protein of $M_r$43,000 (see Figure6.13b), suggesting that unglycosylated sDBH is potentially more susceptible to proteolysis in the absence of its carbohydrate moieties.

## Labelling Cells With [$^3$H]Acetate.

In addition to labelling cells with [$^{35}$S]methionine, some experiments were carried out using [$^3$H]acetate to try and preferentially label cellular fatty acids. The objective was to search for any proteins with covalently attached lipid (e.g. acylated, or bound to phospholipid): in particular I wished to test the hypothesis that the membrane-bound form of DBH might be in this category.

Figure 6.15 shows two-dimensional separations of proteins from cells labelled with [$^3$H]acetate for 2hrs 15min, and for 15 hours. After shorter times of labelling too little radioactivity was incorporated for effective fluorography. However, Figure 6.15 shows that one protein ($M_r$68-70,000) is heavily labelled after a few hours. There is a trace of radioactivity in two or three other proteins, including the putative chromogranin A precursor. After labelling overnight very many components are labelled, presumably a result of the entry of the labelled acetate into cellular amino acid pools. When one compares this labelling pattern with that of methionine labelled cells (Figure 6.5) there are clearly many

Figure 6.15. Cellular Synthesis of Chromaffin Proteins in the Presence of [$^3$H]-Acetate.



(a) (b)

Chromaffin cells ($10^7$cells/ml) were incubated with [$^3$H]-acetate (1.5mCi/ml) in DMEM at 37$^{o}$C in an atmosphere of 95%$O_2$ 5%$CO_2$ for 2.25&18hr. Samples were chilled on ice and solubilised for two-dimensional electrophoresis.

In fluorogram (a) 2.25hr pulse, an $M_r$ 68,000, pI5.8 polypeptide is heavily labelled; the chromogranin A precursor with mature glycosylated material flaring from it was also labelled.

Fluorogram (b) 18hr pulse; a number of additional polypeptides not heavily labelled by methionine are evident in this gel (compare with Figure 6.5b).

Immunoprecipitation studies with antiDBH antiserum indicated that the major labelled protein in (a) was not DBH.

additional polypeptides labelled.

It was of great interest that the component seen in Figure 6.15a runs on the gel in the same position as the major radioactive protein precipitated by anti-DBH serum (Figure 6.14a). However, this heavily labelled component was not precipitated by the anti-DBH antiserum and immunoprecipitated proteins from the 15hr samples (although providing only a very weak signal after extensive exposure) were identical to that of the [$^{35}$S]-methionine labelled proteins.

Discussion.

The chromogranins are the major secretory proteins of the chromaffin cell. They are glycosylated, phosphorylated and sulphated within the Golgi complex, these post-translational modifications affecting their focusing and mobility on two-dimensional gels.

Investigation of protein modification by cell labelling experiments requires the unequivocal identification of the proteins of interest. While immunoprecipitation is the best method for doing this, it proved extremely difficult due to the large cellular content of non-radioactive chromogranins. The immunoprecipitation of radioactive chromogranin A shown in Figure 6.9 is the first successful demonstration that the major radioactive spot seen in cell labelling experiments is indeed this protein. Beyond this I have used the following two criteria for identifying the chromogranins: 1. the positions of radioactive spots on fluorograms have been carefully aligned with Coomassie-stained proteins in the original dried electrophoretograms. 2. All three putative chromogranin precursors were present in gels of microsomal fractions prepared from radiolabelled cells. Furthermore these were protected from digestion by pronase by their enclosure within these microsomal vesicles, characteristic of newly synthesised secretory proteins. These three proteins were the only cell components that could be seen on gels (Figure 6.8) for which protection was clearly demonstrated. Five other proteins, all major labelled components which were associated with microsomes did not appear sensitive to pronase. A pronase resistant digestion product present when microsomal fractions were digested in the presence of Triton X-100 appears to be a fragment of chromogranin A. Although formal

identification will require immunological characterisation, antisera to chromogranin A have been shown to react strongly with a cyanogen bromide resistant product with similar molecular mass isolated from insulin granules (Hutton et al., 1985). The function of chromogranin A is unknown, however the presence of fragments resistant to chemical and enzymatic degradation is these diverse tissues suggests that the study of these products may provide clues to the function of chromogranin A, a protein with a widespread distibution among neuro-endocrine tissues (Somogyi et al., 1984).

In addition to cellular-synthesis studies we have information about the translation products of chromaffin mRNA's in vitro (Kilpatrick, 1985; Falkensammer et al., 1985a&b; Fischer-Colbrie et al., 1986). Two primary translation products more basic than chromogranin A have been identified. These are processed to a single and apparently single polypeptide by the removal of a signal sequence as they are translocated across the membrane and into the lumen of the RER, without any further co-translational modification. This was demonstrated by including dog pancreas microsomal fractions in cell-free translation mixes (Falkensammer et al., 1985a; Kilpatrick et al., 1983). Similarly a 10min pulse with [$^{35}$S]-methionine identifies a chromogranin A precursor whose signal peptide has been removed, and under the appropriate conditions (see Figure 6.7a&b) unprocessed precursor can still be identified by its sensitivity to pronase in microsomal fractions, which processes it to a single and apparently identical spot to that identified in the cell free system in the presence of microsomes. Only 10-30min after the initial pulse with methionine does the chromogranin A precursor begin to show signs of post-translational modifications which markedly affect its electrophoretic behaviour. These modifications

were prevented, while the co-translational removal of the signal peptide was unaffected, by depressing cytosolic ATP levels with the mitochondrial uncoupler FCCP. This suggests that the energy dependent transport of newly synthesised chromogranin A from the RER to the Golgi complex where the post-translational modifications such as O-glycosylation, sulphation and phosphorylation take place (Hanover and Lennarz, 1981), was inhibited. The RER-trapped chromogranin A precursor shows no sign of further modification within this compartment. Consistent with this was the fact that tunicamycin had apparently no significant effect on the biosynthesis of the chromogranins.

A characteristic feature of the chromogranins is that they each display a family of proteolytic breakdown products. These are produced late on during biosythesis since they were not detectable in the RER, as demostrated by blocking transport to the Golgi complex with FCCP, nor were they present after periods of cellular synthesis monitored for up to 18hr. These products are clearly identified in isolated tissue and in cultured cells (Figure 6.3) suggesting that this final modification occurs in the fully matured secretory granule probably over a period of several days (Fleminger et al., 1983).

The processing of chromogranins B and C was consistent with other work (Falkensammer et al., 1985b; Fischer-Colbrie et al., 1986) and exactly parallels the processing of chromogranin A. Chromogranin B showed a larger acidic shift in isoelectric point and this may be due primarily to its sulphation and phosphorylation (Falkensammer, et al., 1985a&b). Sugar analysis (Fischer-Colbrie et al., 1982) and labelling with lectins (Apps et al.,1985) suggest the presence of N-glycosidically linked sugars and this may be supported

here by the attenuation of processing in the presence of tunicamycin. However the reproducibility of this effect was difficult and needs further study, ideally utilising immunoprecipitation.

Chromogranin C has been purified, characterised electrophoretically and synthesised in a cell-free translation system (Fischer-Colbrie et al., 1986). While no carbohydrate analysis has been carried out its cellular synthesis shown here for the first time suggests that like chromogranin A it receives O-linked sugars in the Golgi complex.

Synthesis of the chromogranins was sensitive to monensin. Since no precursor molecules were identified, it can only be assumed that in chromaffin cells this drug arrests the translation of these secretory proteins. The synthesis of cytoplasmic proteins appears unaffected. Since normal precursor chromogranins are not degraded within the RER in the presence of FCCP, it seems unlikley that they have been degraded in the presence of monensin. The preferred site of intervention by monensin in many cell types is the Golgi complex.

**Biosynthesis of Dopamine β-Hydroxylase.**

Studying the biosynthesis of DBH by cellular synthesis and in cell free systems has proved difficult, primarily due to the ease with which putative precursor polypeptides undergo proteolytic degradation during their isolation and analysis. In addition it appears that only de novo synthesis of the soluble form of DBH can be detected, as demonstrated by partitioning of sDBH precursors into the aqueous phase after treatment of fractions with Triton X-114 (L. Kilpatrick and J.G. Pryde unpublished). These problems may account for the lack of concensus in the literature as

to the nature of the DBH precursors.

Until the immunoprecipitation and two-dimensional analysis can be clarified, data shown here for sDBH can only be treated as preliminary. However it does appear that the primary translation product is a polypeptide of $M_r$ 72,000 (sensitive to proteolysis; L. Kilpatrick unpublished) and an identical primary translation product has been reported by Joh et al. (1983). Segregation within the RER is accompanied by an $M_r$ reduction to 67,000 primarily the removal of a signal peptide. This segregation protects the protein from further proteolytic degradation in translation systems.

An identical sDBH precursor ($M_r$ 67,000) is synthesised by cells. As in the cell-free system the protein is sensitive to proteolysis and synthesis in the presence of tunicamycin results in a more highly degraded form of the protein, suggesting that the failure to receive N-linked oligosaccharide has made the sDBH precursor more susceptable to proteolytic degradation. There was no evidence for the biosynthesis of the mature form of sDBH since it could not be identified unequivocally on gels of immunoprecipitated proteins.

# CHAPTER SEVEN

# CONCLUSIONS AND PERSPECTIVES.

Membrane Proteins.

The secretory pathway is discontinuous; there appears to be no physical continuity between membrane bounded compartments. Transport of proteins between them is mediated therefore, by the fission and fusion of membrane vesicles. The Golgi complex lies at the cross-roads of this intracellular traffic in membrane and secretory proteins and the dynamic relationship this organelle has with RER, secretory granules and the plasma membrane has made the study of its own components difficult. When stripped of transitory components its protein fingerprint should reflect its own unique structure and many functions, as has been demonstrated for the chromaffin granule.

Biosynthesis of secretory granule membrane components in the RER and the differentiation of membrane as it passes through the Golgi to secretory granules, poses many questions about how membrane components are selected into stable domains which retain their integrity as they interact with other compartments, such as the plasma membrane, during exocytosis and endocytosis. Subcellular fractionation has been a powerful tool for purifying many organelles and identifying their membrane components. Although, the heterogeneity shown by membranes of microsomal origin suggests that the reticular organelles along the secretory pathway will not be effectively resolved by present fractionation techniques. Fractions of high purity may possibly be isolated by immune-affinity chromatography, if cytoplasmically exposed antigens showing unique localisation can be purified - monoclonal antibodies may have a useful role to play here.

Primary cultures of chromaffin cells have enabled the biosynthesis of the chromogranins to be studied. However the low

level of de novo synthesis of membrane proteins in these cells was unexpected. It may be that the intrinsic technical problems with electrophoresis and immunoprecipitation, encountered with both mDBH and cytochrome $b_{561}$, may be overstating this finding. Therefore further confirmation of this finding with other representative membrane proteins, such as ATPase I and Gp III, may be necessary. However, the absence of precursor proteins to secretory granule membrane proteins of such abundance in both isolated cells and tissue strongly suggests that there is indeed only very low levels of de novo synthesis in this fully differentiated cell.

Many chromaffin granule membrane proteins were present to varying degrees in the subcellular fractions isolated here. To what extent they were present only as contaminants was hard to estimate. In the absence of unequivocal marker enzyme analysis this could not be quantified effectively. In future the accurate determination of mDBH activity from cell fractions may be possible by phase separation in Triton X-114 - it may even be possible to detect its biosynthetic precursors by the judicious use of lectin-affinity chromatography.

The assembly of chromaffin granule membrane from newly synthesised components which have undergone post-translational modifications within the Golgi complex has still to be formally demonstrated. The similarities shown however between the phospholipid-rich phases of Golgi and chromaffin granule membranes and the differences in terminal glycosylation states between glycoproteins in the RER fraction and those in the Golgi and chromaffin granule membranes, suggest that the biosynthesis and assembly of the granule membrane follows the same pathway along which viral envelope glycoproteins trespass on their way to the

plasma membrane. The focus of attention may best be turned to the biosynthesis of ATPase I and the membrane glycoproteins Gp II, III & IV.

The purification and production of antibodies to these proteins must therefore be a priority. The Triton X-114 separation protocol developed in this thesis has already enabled ATPase I to be identified, purified and characterised (Percy et al., 1985; Percy and Apps, 1986) and antibodies, while proving difficult to raise, should be available soon. This separation protocol also provides material for the purification of the membrane glycoproteins. To complement the biochemical characterisation of these antigens their cellular localisation should be demonstrated by immunofluorescence and immunoelectron microscopy.

While analysis of membrane protein biogenesis was frustrated by their low turnover in chromaffin cells, these results are consistent with data that suggest that secretory granule membranes are reutilised to package newly synthesised secretory proteins following exocytosis (Winkler, 1977).

## Secretory Proteins.

The biosynthesis of the chromogranins is now well established. They are synthesised on membrane-bound polysomes, segregated within the RER, then transported to the Golgi complex for post-translational modification before being packaged for secretion. The molecular nature of their transport between compartments is the next exciting goal. I have shown that transport between the RER and Golgi complex can be inhibited by the mitochondrial uncoupler FCCP. It remains to be demonstrated that this is reversible (Datema and Schwarz, 1981). The stability of the chromogranin A precursor

within the RER membrane fractions, for which a fractionation protocol has been described here, provides the potential for a cell-free assay to study the molecular basis of transport of secretory proteins from the RER to the Golgi complex. Chromaffin cells radiolabelled in the presence of FCCP would provide donor RER membranes, while CHO cell Golgi membranes and cytosol, which have been used successfully to study inter-Golgi transport (Balch et al., 1984a&b), may act as acceptors and provide the cofactors involved. Immunoprecipitation of chromogranin A and analysis by two-dimensional electrophoresis, would provide a less complicated system than the use of N-linked secretory or viral glycoproteins which require acquisition of endo H resistance to demonstrate transport to the Golgi complex.

Membrane Recycling.

How is secretory granule membrane recycled after exocytosis, to package newly synthesised secretory proteins? There is currently a great amount of effort being put into the development of cell-free systems to study endocytosis. Chromaffin cells provide a good model for studying endocytosis since this process can be stimulated by exposing cells to secretogogues. Mapping the pathway taken by recycled chromaffin granule membrane proteins, transiently exposed at the cell surface, by labelling with $^{125}I$, and tracking the fate of individual proteins by immunoprecipitation to study the kinetics of this transport will be the first step in embarking upon cell-free reconstitution studies.

The plasma membrane of the chromaffin cell has yet to be properly characterised. The highly purified fraction, albeit of low abundance isolated here from microsomal fractions may provide a

start. Present evidence tentitively assigns this fraction a plasma membrane origin. However its exact localisation remains to be demonstrated. A priority must be the production of antibodies to the lentil lectin binding protein ($M_r$ 32,000) which is abundant in this fraction; fluorescence microscopy should provide a rapid answer to its location. It cannot be ruled out however, that this fraction is derived from another organelle such as the Golgi complex (or endosomes).

Recently the chromaffin cell succumbed to the attentions of molecular biology with the publication of the genomic sequence for chromogranin A. Molecular genetics should provide the tools necessary for the initial dissection of the exocytotic and endocytotic pathways. For the bovine chromaffin cell to continue to remain an avant-coureur for cell biology the establishment of transformed cell lines and mutants deficient in components of these pathways will be required for their molecular dissection.

REFERENCES.

Abbs, M.T., and Phillips, J.H. (1980) Biochim. Biophys. Acta **595:**200-221.

Aberer, W., Kostron, H., Huber, E., and Winkler, H. (1978) Biochem. J. **172:**353-360.

Abrahams, S.J., and Holtzman, E. (1973) J. Cell Biol. **56:**540-558.

Altstiel, L., and Branton, D. (1983) Cell **32:**921-929.

Amsterdam, A., and Jamieson, J.D. (1974) J. Cell Biol. **63:**1057-1073.

Amsterdam, A., Schramm, M.M., Ohad, I., Salomon, Y. and Selinger, Z. (1971) J. Cell Biol. **50:**187-200.

Anderson, D.J., and Blobel, G. (1983) Methods Enzymol. **96:**111-120.

Apps, D.K., Boisclair, M.D., Gavine, F.S., and Pettigrew, G.W. (1984) Biochim. Biophys. Acta **764:**8-16.

Apps, D.K., Phillips, J.H., and Purves, F.C. (1985) Neuroscience **16:**477-487.

Apps, D.K., Pryde, J.G., and Phillips, J.H. (1980a) Neuroscience **5:**2279-2287.

Apps, D.K., Pryde, J.G., and Phillips, J.H. (1980b) FEBS Lett. **111:**386-390.

Apps, D.K., Pryde, J.G., Sutton, R., and Phillips, J.H. (1980c) Biochem. J. **190:**273-282.

Apps, D.K., Pryde, J.G., Sutton, R. (1982) Annals N.Y. Acad. Sci. **402:**134-145.

Apps, D.K., Pryde, J.G., Sutton, R. (1983) Neuroscience **9:**687-700.

Apps, D.K., and Reid, G.A. (1977) Biochem. J. **167:**297-300.

Apps, D.K., and Schatz, G. (1979) Eur. J. Biochem. **100:**411-419.

Arthur, G., and Sheltawy, A. (1980) Biochem. J. **191:**523-532.

Ash, D.E., Papadopoulos, N.J., Colombo, G., and Villafranca, J.J. (1984) J. Biol. Chem. **259:**3395-3398.

Atkinson, P.H., and Lee, J.T. (1984) J. Cell Biol. **98:**2245-2249.

Aunis, D., Guerold, B., Bader, M.F., and Ciesielski-Treska, J. (1980) Neuroscience **5**:2261-2277.

Avruch, J., and Wallach, D.F.H. (1971) Biochim. Biophys. Acta **233**:334-347.

Babad, H., and Hassid, W.Z. (1966) Methods Enzymol. **8**:346-351.

Baeuerle, P.A., and Huttner, W.B. (1984) The EMBO J. **3**:2209-2215.

Balch, W.E., Dunphy, W.G., Braell, W.A., and Rothman, J.E. (1984a) Cell **39**:405-416.

Balch, W.E., Glick, B.S. and Rothman, J.E. (1984b) Cell **39**:525-536.

Banks, P. (1965) Biochem. J. **95**:490-496.

Bartles, J.R., Braiterman, L.T., and Hubbard, A.L. (1985) J. Biol Chem. **260**:12792-12802.

Bartlett, G.R. (1959) J. Biol. Chem. **234**:466-468.

Bashford, C.L. Radda, G.K., and Ritchie, G.A. (1975a) FEBS Lett. **50**:21-24.

Bashford, C.L. Casey, R.P., Radda, G.K., and Ritchie, G.A. (1975b) Biochem. J. **148**:153-155.

Baumgartner, H., Gibb, J.W., Hörtnagl, H., Snider, S.R., and Winkler, H. (1974) Mol. Pharmacol. **10**:678-685.

Benedum, U.M., Baeuerle, P.A., Konecki, D.S., Frank, R., Powell, J., Mallet, J., and Huttner, W.B. (1986) The EMBO J. **5**:1495-1502.

Berger, M. and Schmidt, M.F.G. (1984) The EMBO J. **3**:713-719.

Bergeron, J.J.M., Borts, D., and Cruz, J. (1978) J. Cell Biol. **76**:87-97.

Bergeron, J.J.M., Ehrenreich, J.H., Siekevitz, P., and Palade, G.E. (1973) J. Cell Biol. **59**:73-88.

Bergeron, J.J.M., Kotwal, G.J., Levine, G., Bilan, P., Rachubinski, R., Hamilton, M., Shore, G.C., and Ghosh, H.P. (1982) J. Cell Biol. **94**:36-41.

Bergmann, J.E., Tokuyasu, K.T., and Singer, S.J. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**:1746-1750.

Berlin, R.D., and Oliver, J.M. (1980) J. Cell Biol. **85**:660-671.

Berlin, R.D., Oliver, J.M., and Walter, R.J. (1978) Cell **15:**327-341.

Bernard, C. (1865) An Introduction to the Study of Experimental Medicine. (Greene, G.H.C. (1937) trans.) Dover edition (1957), Dover Publications, Inc. New York.

Beyer, T.A., Rearick, J.I., Paulson, J.C., Prieels, J-P., Sadler, J.E., and Hill, R.L. (1979) J. Biol. Chem. **254:**12531-12541.

Bischoff, J., and Kornfeld, R. (1983) J. Biol. Chem. **258:**7907-7910.

Blaschko, H. (1939) J. Physiol. **96:**50P-51P.

Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. **77:**1496-1500.

Blobel, G., and Potter, V.R. (1968) Biochim. Biophys. Acta **166:**48-57.

Blobel, G., Walter, P., Chang, C., Goldman, B., Erickson, A., and Lingappa, V.R. (1979) Symp. Soc. Exp. Biol. **33:**9-36.

Bordier, C. (1981) J. Biol. Chem. **256:**1604-1607.

Bottenstein, J.E., and Sato, G.H. (1979) Proc. Natl. Acad. Sci. U.S.A. **76:**514-517.

Bradford, M.M. (1976) Anal. Biochem. **72:**248-254.

Braell, W.A., Balch, W.E.. Dobbertin, D.C., and Rothman, J.E. (1984) Cell **39:**511-524.

Bretz, R., Bretz, H., and Palade, G.E. (1980) J. Cell Biol. **84:**87-101.

Bretz, R., and Staubli, W. (1977) Eur. J. Biochem. **77:**181-192.

Brown, W.J., and Farquhar, M.G. (1984) Cell **36:**295-307.

Buckley, K., and Kelly, R.B. (1985) J. Cell Biol. **100:**1284-1294.

Burgoyne, R.D. (1984) Biochim. Biophys. Acta **779:**201-216.

Burgun, C., Martinez De Munoz, D., and Aunis, D. (1985) Biochim. Biophys. Acta **839:**219-227.

Burke, B., Griffiths, G., Reggio, H., Louvard, D., and Warren, G. (1982) The EMBO J. **1:**1621-1628.

Burns, D.M., and Touster, O. (1982) J. Biol. Chem. **257:**9991-10000.

Cahill, A.L., and Morris, S.J. (1979) J. Neurochem. **32:**855-867.

Carey, D.J., and Hirschberg, C.B. (1980) J. Biol. Chem. **255:**4348-4354.

Castle, J.D., Jamieson, J.D., and Palade, G.E. (1972) J. Cell Biol. **53:**290-311.

Castle, J.D., Jamieson, J.D., and Palade, G.E. (1975) J. Cell Biol. **64:**182-210.

Ceccarelli, B., Clemente, F., and Meldolesi, J. (1975) J. Physiol. **245:**617-638.

Chamberlain, J.P. (1979) Anal. Biochem. **98:**132-135.

Cheetham, R.D., Morre, D.J., and Yunghans, W.N. (1970) J. Cell Biol. **44:**492-500.

Cheetham, R.D., Morre, D.J., Pannek, C., and Friend, D.S. (1971) J. Cell Biol. **49:**899-905.

Cidon, S., Ben-David, H., and Nelson, N. (1983) J. Biol. Chem. **258:**11684-11688.

Cidon, S., and Nelson, N. (1983) J. Biol. Chem. **258:**2892-2898.

Clemetson, K.J., Bienz, D., Zahno, M.-L., and Luscher, E.F. (1984) Biochim. Biophys. Acta **778:**463-469.

Coates, S.W., Gurney, T., Sommers, L.W., Yeh, M., and Hirschberg, C.B. (1980) J. Biol. Chem. **255:**9225-9229.

Cohn, D.V., Zangerle, R., Fischer-Colbrie, R., Chu, L.L.H., Elting, J.J., Hamilton, J.W., and Winkler, H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79:**6056-6059.

Corcoran, J.J., Lattanzio, F.A., Rubin, R.W. and Pressman, B.C. (1982) Biochim. Biophys. Acta **707:**226-235.

Coupland, R.E. (1965) J. Anat. **99:**231-254.

Coupland, R.E., and Kobayashi, S. (1976) In: Chromaffin, Enterochromaffin and Related Cells (eds. Coupland, R.E. and Fujita, T.), pp.59-81. Elsevier, Amsterdam.

Crompton, M., Kunzi, M., and Carafoli, E. (1977) Eur. J. Biochem. **79:**549-558.

Daniels, A., Korda, A., Tanswell, P., Williams, A. and Williams, R.J.P. (1974) Proc. R. Soc. Lond. B. **187:**353-361.

Da Prada, M., Pletscher, A., and Tranzer, J.P. (1972) Biochem. J. **127:**681-683.

Datema, R., and Schwarz, R.T. (1981) J. Biol. Chem. **256:**11191-11198.

Davis, J.G., Mapes, C.J., and Donovan, J.W. (1971) Biochemistry **10:**39-42.

Davis, N.G. and Model, P. (1985) Cell **41:**607-614.

De Duve, C. (1975) Science (Wash.) **189:**186-194.

De Oliveira-Filgueiras, O.M., Van Den Boch, H., Johnson, R.G., Carty, S.E., and Scarpa, A. (1981) FEBS Lett. **129:**309-313.

De Oliveira-Filgueiras, O.M., Van Den Besselaar, A.M.H.P., and Van Den Bosch, H. (1979) Biochim. Biophys. Acta **558:**73-84.

Diliberto, E.J., and Allen, P.L. (1981) J. Biol. Chem. **256:**3385-3393.

Distler, J., Hieber, V., Sahagian, G., Schmickel, R., and Jourdian, G.W. (1979) Proc. Natl. Acad. Sci. U.S.A. **76:**4235-4239.

Docherty, K., Carroll, R.J., and Steiner, D.F. (1982) Proc. Natl. Acad. Sci. U.S.A. **79:**4613-4617.

Dottavio-Martin, D. and Ravel, J.M. (1978) Anal. Biochem. **87:**562-565.

Douglas, W.W. (1968) Brit. J. Pharmacol. **34:**451-474.

Douglas, M.G., and Butow, R.A. (1973) Proc. Natl. Acad. Sci. U.S.A. **73:**1083-1086.

Dowd, D.J., Edwards, C., Englert, D., Mazurkiewicz, J.E., and Ye, H.Z. (1983) Neuroscience **10:**1025-1033.

Duch, D.S., Viveros, O.H., and Kirshner, N. (1968) Biochem. Pharmacol. **17:**233-264.

Duerr. A.C., Guluzian, L., and Trifaró, J.M. (1974) Pharmacologist **16:**276.

Dunphy, W.G., Fries, E., Urbani, L.J., and Rothman, J.E. (1981) Proc. Natl. Acad. Sci. U.S.A. **78:**7453-7457.

Duong, L.T., and Fleming, P.J. (1982) J. Biol. Chem. **257:**8561-8564.

Duong, L.T., and Fleming, P.J. (1984) Arch. Biochem. Biophys. **228:**332-341.

Ehrenreich, J.H., Bergeron, J.M., Siekevitz, P., and Palade, G.E. (1973) J. Cell Biol. **59:**45-72.

Falkensammer, G., Fischer-Colbrie, R., Richter, K., and Winkler, H. (1985a) Neuroscience **14:**735-746.

Falkensammer, G., Fischer-Colbrie, R., and Winkler, H. (1985b) J.Neurochem. **45:**1475-1480.

Farquhar, M.G. (1978) J. Cell Biol. **77:**R35-R42.

Farquhar, M.G. (1982) In: Membrane Recycling, Ciba Found. Symp. (Evered, D., and Collins, G.M. eds.) vol, 92 pp.174-183. Pittman Press, Bath.

Farquhar, M.G., and Palade, G. (1981) J. Cell Biol. **90:**77S-103S.

Fenwick, E.M., Fajdiga, P.D., Howe, N.B.S., and Livett, B.G. (1978) J. Cell Biol. **76:**12-30.

Fischer-Colbrie, R., and Frischenschlager, I. (1985) J. Neurochem. **44:**1854-1861.

Fischer-Colbrie, R., Hagn, C., Kilpatrick, L. and Winkler, H. (1986) J. Neurochem. **47:**318-321.

Fischer-Colbrie, R., Schachinger, M., Zangerle, R., and Winkler, H. (1982) J. Neurochem. **38:**725-732.

Fischer-Colbrie, R., Zangerle, R., Frischenschlager, I., Weber, A., and Winkler, H. (1984) J. Neurochem **42:**1008-1016.

Fisher, R.J., Liang, A.M., and Sundstrom, G.C. (1981) J. Biol. Chem. **256:**707-715.

Fitting, T., and Kabat, D. (1982) J. Biol Chem. **257:**14011-14017.

Flatmark, T., and Gronberg, M. (1981) Biochem. Biophys. Res. Commun. **99:**292-301.

Flatmark, T., Lagercrantz, H., Terland, O., Helle, K.B., and Stjarne, L. (1971) Biochim. Biophys. Acta **245:**249-252.

Fleischer, B. (1981) J. Cell Biol. **89:**246-255.

Fleischer, B., Fleischer, S., and Ozawa, H. (1969) J. Cell Biol. **43:**59-79.

Fleischer, B., and Fleischer, S. (1970) Biochim. Biophys. Acta **219:**301-319.

Fleminger, G., Ezra, E., Kilpatrick, D.L. and Udenfriend, S. (1983) Proc. Natl. Acad. Sci. U.S.A. **80:**6418-6421.

Forgac, M., Cantley, l., Wiedenmann, B., Alstiel, L., and Branton, D. (1983) Proc. Natl. Acad. Sci. U.S.A. **80:**1300-1303.

Friedman, S., and Kaufman, S. (1965) J. Biol. Chem. **240:4763-4773.**

Friend, D.S., and Murray, M.J. Amer. J. Anat. **117:**135-150.

Fries, E., Gustafsson, L., and Peterson, P.A. (1984) The EMBO J. **3:**147-152.

Gabel, C.A., and Kornfeld, S. (1982) J. Biol. Chem. **257:**10605-10612.

Gagnon, C., Schatz, R., Otten, U., and Thoenen, H. (1976) J. Neurochemistry **27:**1083-1089.

Garewal, H.S. (1973) Anal. Biochem. **54:**319-324.

Gavine, F.S., Pryde, J.G., Deane, D.L., and Apps, D.K. (1984) J. Neurochem. **43:**1243-1252.

Geisow, M.J., and Burgoyne, R.D. (1983) Nature (Lond.). **301:**432-435.

Gerber, D., Davis, M., and Hokin, L.E. (1973) J. Cell Biol. **56:**736-745.

Geuze, J.J., and Kramer, M.F. (1974) Cell Tiss. Res. **156:**1-20.

Gibson, R., Kornfeld, S., and Schlesinger, S. (1980) Trends Biochem. Sci. **5:**290-293.

Gilmore, R., Blobel, G., and Walter, P. (1982a) J. Cell Biol. **95:**463-469.

Gilmore, R., Blobel, G., and Walter, P. (1982b) J. Cell Biol. **95:**470-477.

Glass, W.F., Briggs, R.C., and Hnilica, L.S. (1981) Anal. Biochem. **115:**219-224.

Glickman, J., Croen, K., Kelly, S., and Al-Awqati, Q. (1983) J. Cell Biol. **97:**1303-1308.

Godelaine, D., Spiro, M.J., and Spiro, R.G. (1981) J. Biol. Chem. **256:**10161-10168.

Goldberg, D.E., and Kornfeld, S. (1983) J. Biol. Chem. **258:**3159-3165.

Green, J., Griffiths, G., Louvard, D., Quinn, P., and Warren, G.B. (1981) J. Mol. Biol. **152:**663-698.

Griffiths, G., Brands, R., Burke, B., Louvard, D., and Warren, G. (1982) J. Cell Biol. **95:**781-792.

Grouselle, M., and Phillips, J.H. (1982) Biochem. J. **202:**759-770.

Grueninger, H.A., Apps, D.K., and Phillips, J.H. (1983) Neuroscience **9:**917-924.

Guan, J.-L., and Rose, J.K. (1984) Cell **37:**779-787.

Hanover, J.A. and Lennarz, W.J. (1981) Arch. Biochem. Biophys. **211:**1-19.

Hanover, J.A., Lennarz, W.J., and Young, J.D. (1980) J. Biol. Chem. **255:**6713-6716.

Harwood, R., Grant, M.E., and Jackson, D.S. (1976) Biochem. J. **156:**81-90.

Hartree, E.F. (1972) Anal. Biochem. **48:**422-427.

Harnadek, G.J., Callahan, R.E., Barone, A.R., and Njus, D. (1985) Biochemistry **24:**384-389.

Häusler, R., Burger, A. and Niedermier, W. (1981) Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmacol. **315:**255-267.

Hawkes, R. (1982) Anal. Biochem. **123:**143-146.

Helenius, A., and Simons, K. (1975) Biochim. Biophys. Acta **415:**29-79.

Helle, K.B. (1971) Biochim. Biophys. Acta **245:**94-104.

Helle, K.B., Serck-Hanssen, G., and Bock, E. (1981) In: Chemical Neurotransmission 75 Years (eds. Stjarne, L., Lagercrantz, H., Hedqvist, P., and Wennmalm, A.), pp.85-90. Academic Press, London.

Hercz, A., and Harpaz, N. (1980) Can. J. Biochem. **58:**644-648.

Herzog, V., and Farquhar, M.G. (1977) Proc. Natl. Acad. Sci. U.S.A. **74:**5073-5077.

Hesketh, T.R., Beaven, M.A., Rogers, J., Burke, B., and Warren, G.B. (1984) J. Cell Biol. **98:**2250-2254.

Hettkamp, H., Legler, G., and Bause, E. (1984) Eur. J. Biochem. **142:**85-90.

Higgins, J.A. (1984) Biochem. J. **219:**261-272.

Hillarp, N-A. (1959) Acta Physiol. Scand. **47:**271-279.

Hoflack, B., Cacan, R., Verbert, A. (1981) Eur. J. Biochem. **117:**285-290.

Hogue-Angeletti, R.A. (1977) Arch. Biochem. Biophys. **184:**364-372.

Holtzman, E. (1977) Neuroscience **2:**327-355.

Holtzman, E., Schachte, S., Evans, J., and Treichbert, S. (1978) In: Cell Surface Reviews, (G. Poste and G.L. Nicholson eds) vol **4** pp.166-246. Elsevier, Amsterdam.

Hörtnagl, H. (1976) Neuroscience **1:**9-18.

Hörtnagl, H., Winkler, H., and Lochs, H. (1972) Biochem. J. **129:**187-195.

Hörtnagl, H., Winkler, H., Schopf, J.A.L., and Hohenwallner, W. (1971) Biochem. J. **122:**299-304.

Houstek, J., Pavelka, S., Kopecky, J., Drahota, Z., and Palmieri, F. (1981) FEBS Lett. **130:**137-140.

Howell, J.I., and Lucy, J.A. (1969) FEBS Lett. **4:**147-150.

Howell, K.E. and Palade, G.E. (1982) J. Cell Biol. **92:**822-832.

Hubbard, S.C., and Ivatt, R.J. (1981) Ann. Rev. Biochem. **50:**555-583.

Huber, E., Konig, P., Schuler, G., Aberer, W., Plattner, H., and Winkler, H., (1979) J. Neurochem. **32:**35-47.

Hunter, A., Waldron, K., and Apps, D.K. (1982) FEBS Lett. **144:**51-56.

Huttner, W.B. (1982) Nature (Lond.) **299:**273-276.

Huttner, W.B. (1984) Methods Enzymol. **107:**200-223.

Hutton, J.C., Hansen, F., and Peshavaria, M. (1985) FEBS Lett. **188:**336-340.

Ichikawa, Y., and Yamano, T. (1965) Biochem. Biophys. Res. Commun. **20**:263-268.

Ingebretsen, O.C., Terland, O., and Flatmark, T. (1980) Biochim. Biophys. Acta **628**:182-189.

Ito, A., and Palade, G.E. (1978) J. Cell Biol. **79**:590-597.

Jacobs, L.S., McKeel, D.W., Jarret, L., and Daughaday, W.H. (1973) Endocrinology **92**:477-486.

Jamieson, J.D., and Palade, G.E. (1968) J. Cell Biol. **39**:589-603.

Jeffery, P.L., Brown, D.H., and Brown, B.I. (1970) Biochemistry **9**:1403-1415.

Joh, T.H., Baetge, E.E., Ross, M.E., and Reis, D.J. (1983) Cold Spring Harbour Symp. Quant. Biol. **48**:327-335.

Johnson, R.G., and Scarpa, A. (1976) J. Gen. Physiol. **68**:601-631.

Johnson, R.G., and Scarpa, A. (1979) J. Biol. Chem. **254**:3750-3760.

Johnson, D.C., and Spear, P.G. (1983) Cell **32**:987-997.

Kalina, M., and Robinovitch, R. (1975) Cell Tiss. Res. **163**:373-382.

Kanagasuntherm, P., and Lim, S.C. (1981) J. Physiol. **312**:445-454.

Kanner, B.I., Sharon, I., Maron, R., and Schuldiner, S. (1980) FEBS Lett. **111**:83-86.

Kaplan, F., and Hechtman, P. (1983) J. Biol. Chem. **258**:770-776.

Katz, F.N., Rothman, J.E., Lingappa, V.R., Blobel, G., and Lodish, H.F. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**:3278-3282.

Kelly, R.B. (1985) Science 230:25-32.

Kiang, W-L., Krusius, T., Finne, J., Margolis, R.U., and Margolis, R.K. (1982) J. Biol. Chem. **257**:1651-1659.

Kilpatrick, L. (1985) Phd. Thesis, University of Edinburgh.

Kilpatrick, L., Gavine, F., Apps, D.K., and Phillips, J.H. (1983) FEBS Lett. **164**:383-388.

Kirshner, N. (1962) J. Biol. Chem. **237**:2311-2317.

Kirshner, N. (1974) In: Advances in Cytopharmacology (Ceccarelli, B., Clemente, F., and Meldolesi, J. eds.) vol 2 pp265-272. Raven Press, New York.

Kirshner, N. (1975) In: Adrenal Gland. Handbook of Physiology (eds. Blaschko, H., Sayers, G. and Smith, A.D.), vol.6 pp.341-355. American Physiological Soc., Bethesda.

Klinman, J.P., Krueger, M., Brenner, M., and Edmondson, D.E. (1984) J. Biol. Chem. **259**:3399-3402.

Knoth, J., Handloser, K., and Njus, D. (1980) Biochemistry **19**:2938-2942.

Koerker, R.L., Hahn, W.E., and Schneider, F.H. (1974) Eur. J. Pharmacol. **28**:350-359.

Koike, H., and Meldolesi, J. (1981) Exp. Cell Res. **134**:377-388.

Kopell, W.N. and Westhead, E.W. (1982) J. Biol. Chem. **257**:5707-5710.

Kornfeld, R., and Kornfeld, S. (1985) Ann. Rev. Biochem. **54**:631-664.

Krieger-Brauer, H., and Gratzl, M. (1982) Biochim. Biophys. Acta **691**:61-70.

Kruggel, W., O'Connor, D.T., and Lewis, R.V. (1985) Biochem. Biophys. Res. Commun. **127**:380-383.

Laemmli, U.K., and Favre, M. (1973) J. Mol. Biol. **80**:575-599.

Lane, N., Caro, L., Otero-Vilardebo, L.R., and Goodman, G.C. (1964) J. Cell Biol. 21:339-351.

LeBel, D., Poirier, G.G., and Beaudoin, A.R. (1978) Anal. Biochem. **85**:86-89.

Ledbetter, F.H., Kilpatrick, D., Sage, H.L., and Kirshner, N. (1978) Am. J. Physiol. 235:E475-E486.

Ledford, B.E. and Davis, D.F. (1983) J. Biol. Chem. **258**:3304-3308.

Leis, J.F., and Kaplan, N.O. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**:6507-6511.

Lingappa, V.R., Lingappa, J.R., Prasad, R., Ebner, K.E., Blobel, G. (1978) Proc. Natl. Acad. Sci. U.S.A. 75:2338-2342.

Lingg, G., Fischer-Colbrie, R., Schmidt, W. and Winkler, H. (1983) Nature (Lond.). 301:610-611.

Livett, B.G., Kozousek, V., Mizobe, F., and Dean, D.M. (1979) Nature (London) 278:256-257.

Ljones, T., Skotland, T., and Flatmark, T. (1976) Eur. J. Biochem. 61:525-533.

Lodish, H.F. and Kong, N. (1984) J. Cell Biol. 98:1720-1729.

Lodish, H.F., Kong, N., Snider, M. and Strous G.J.A.M. (1983) Nature (Lond.) 304:80-83.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193:265-275.

Magee, A.I., and Courtneidge, S.A. (1985) The EMBO J. 4:1137-1144.

Maher, P.A., and Singer, S.J. (1985) Proc. Natl. Acad. Sci. U.S.A. 82:958-962.

Majzoub, J.A., Dee, P.C., and Habener, J.F. (1982) J. Biol. Chem. 257:3581-3588.

Margolis, R.K., Finne, J., Krusius, T., and Margolis, R.U. (1984) Arch. Biochem. Biophys. 228:443-449.

Mason, T.L. Poyton, R.O., Wharton, D.C., and Schatz, G. (1973) J. Biol. Chem. 248:1346-1354.

Massey, V. (1959) Biochim. Biophys. Acta 34:255-256.

Masur, S.K., Holtzman, E., and Walter, R. (1972) J. Cell Biol. 52:211-219.

Matlin, K., and Simons, K. (1983) Cell 34:233-243.

Matthew, W.D., Tsavaler, L., and Reichardt, L.F. (1981) J. Cell Biol. 91:257-269.

McGuire, E.J., Jourdian, G.W., Carlson, D.M., and Roseman, S. (1965) J. Biol. Chem. 240:PC4112-4115.

McHugh, E.M., McGee, R., and Fleming, P.J. (1985) J. Biol. Chem. 260:4409-4417.

McKeel, D.W., and Jarett, L. (1974) J. Cell Biol. 62:231-236.

Meldolesi, J. (1974a) J. Cell Biol. 61:1-13.

Meldolesi, J. (1974b) In: Cytopharmacology of Secretion. (Ceccarelli, B., Clemente, F., and Meldolesi, J. eds.), vol. 2 pp.71-86. Raven Press, New York.

Meldolesi, J., Borgese, N., De Camilli, P., and Ceccarelli, B. (1978) In: Cell Surface Reviews (G. Poste and G. Nicholson, eds), vol.5 pp.510-629. North-Holland Publ. Amsterdam.

Meldolesi, J., and Ceccarelli, B. (1981) Phil. Trans. R. Soc. Lond. **296**:55-65.

Meldolesi, J., and Cova, D. (1971) J. Cell Biol. **51**:396-404.

Meldolesi, J., and Cova, D. (1972) J. Cell Biol. **55**:1-18.

Meyer, D.I., Krause, E., and Dobberstein, B. (1982a) Nature (Lond.). **297**:647-650.

Meyer, D.I., Louvard, D., and Dobberstein, B. (1982b) J. Cell Biol. **92**:579-583.

Michael, J.M., and Kornfeld, S. (1980) Arch. Biochem. Biophys. **199**:249-258.

Michell, R.H., and Hawthorne, J.N. (1965) Biochem. Biophys. Res. Commun. **21**:333-338.

Montgomery, R., and Wu, Y-C. (1963) J. Biol. Chem. **238**:3547-3554.

Morgan, E.H., and Peters, T. (1971) J. Biol. Chem. **246**:3508-3511.

Morre, D.J. (1971) Methods Enzymol. **22**:130-148.

Morre, D.J., Hamilton, R.L., Mollenhauer, H.H., Mahley, R.W., Cunningham, W.P., Cheetham, R.D., and Lequire, U.S. (1970) J. Cell Biol. **44**:484-491.

Morre, D.J., Merlin, L.M., and Keenan, T.W. (1969) Biochem. Biophys. Res. Commun. **37**:813-819.

Morre, D.J., and Mollenhauer, H.H. (1964) J. Cell Biol. **23**:295-305.

Mundy, D.I. and Strittmatter, W.J. (1985) Cell **40**:645-656.

Munro, H.N., and Fleck, A. (1966) Analyst **91**:78-88.

Nagasawa, J., Douglas, W.W., and Schulz, R.A. (1971) Nature **232**:341-342.

Nagasawa, J., and Douglas, W.W. (1972) Brain Res. **37**:141-145.

Natowicz, M.R., Chi, M.M-Y., Lowry, O.H., and Sly, W.S. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**:4322-4326.

Neutra, M., and Leblond, C.P. (1966a) J. Cell Biol. **30:**119-136.

Neutra, M., and Leblond, C.P. (1966b) J. Cell Biol. **30:**137-150.

Niedermier, W., and Burger, A. (1981) Naunyn-Schmiedebergs Arch. Pharmacol **316:**69-80.

Niemann, H., Boschek, B., Evans, D., Rosing, M., Tamura, T., and Klenk, H.-D. (1982) The EMBO J. **1:**1499-1504.

Nijjar, M.S. and Hawthorne, J.N. (1974) Biochim. Biophys. Acta **367:**190-201.

Njus, D., Knoth, J., Cook, C. and Kelley, P.M. (1983) J. Biol. Chem. **258:**27-30.

Njus, D., Knoth, J., and Zallakian, M. (1981) Curr. Top. Bioenerg. **11:**107-147.

Njus, D., and Radda, G.K. (1978) Biochim. Biophys. Acta **463:**219-244.

Njus, D., and Radda, G.K. (1979) Biochem. J. **180:**579-585.

Nolan, J., Fonesca, R., and Hogue-Angeletti, R. (1985) Arch. Biochem. Biophys. **240:**257-264.

Novikoff, A.B. (1976) Proc. Natl. Acad. Sci. U.S.A. **73:**2781-2787.

Novikoff, P.M., Novikoff, A.B., Quintana, N., and Hauw, J.-J. (1971) J. Cell Biol. **50:**859-886.

O'Connor, D.T., and Frigon, R.P. (1984) J. Biol. Chem. **259:**3237-3247.

O'Farrell, P.H. (1975) J. Biol. Chem. **250:**4007-4021.

Ohkuma, S., and Poole, B. (1978) Proc. Natl. Acad. Sci. U.S.A. **75:**3327-3331.

Olden, K., Pratt, R.M., and Yamada, K. (1978) Cell **13:**461-473.

Omura, T., and Sato, R. (1964) J. Biol. Chem. **239:**2370-2378.

Orci, L., Glick, B.S., and Rothman, J.E. (1986) Cell **46:**171-184.

Orci, L., Halban, P., Amherdt, M., Ravazzola, M., Vassalli, J.-D., and Perrelet, A. (1984) Cell **39:**39-47.

Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Vassalli, J.-D., and Perrelet, A. (1985) Cell 42:671-681.

Oseroff, A.R., Robbins, P.W., and Burger, M.M. (1973) Ann. Rev. Biochem. 42:647-682.

Ottosen, P.D., Courtoy, P.J., and Farquhar, M.G. (1980) J. Exp. Med. 152:1-19.

Palade, G.E. (1975) Science (N.Y.) 189:347-358.

Palade, G.E. (1959) In: Subcellular Particles (Hayashi T. ed.), pp.64-70. Ronald Press, New York.

Parker, F., and Peterson, N.F. (1965) J. Lipid Res. 6:455-460.

Parks, D.R., Bryan, V.M., Oi, V.T., and Herzenberg, L.A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:1962-1966.

Parodi, A.J., Mendelzon, D.H., Lederkremer, G.Z., and Martin-Barrientos, T. (1984) J. Biol. Chem. 259:6351-6357.

Patzak, A., and Winkler, H. (1986) J. Cell Biol. 102:510-515.

Patzak, A., Bock, G., Fischer-Colbrie, R., Schauenstein, K., Schmidt, W., Lingg, G., and Winkler, H. (1984) J. Cell Biol. 98:1817-1824.

Pearse, B.M.F. (1976) Proc. Natl. Acad. Sci. U.S.A. 73:1255-1259.

Peer, L.J., Winkler, H., Snider, S.R., Gibb, J.W., and Baumgartner, H. (1976) Biochem. Pharmacol. 25:311-315.

Pelletier, G. (1973) J. Ultrastructure Res. 43:445-459.

Percy, J.M., and Apps, D.K. (1986) Biochem, J. 239:77-81.

Percy, J.M., Pryde, J.G., and Apps, D.K. (1985) Biochem. J. 231:557-564.

Perez, M., and Hirschberg, C.B. (1985) J. Biol. Chem. 260:4671-4678.

Phillips, J.H. (1973) Biochem. J. 136:579-587.

Phillips, J.H. (1981) Biochem. J. 200:99-107.

Phillips, J.H., Allison, Y.P., and Morris, S.J. (1977) Neuroscience 2:147-152.

Phillips, J.H., and Apps, D.K. (1979) In: Physiological and Pharmacological Biochemistry, International Review of Biochemistry (Tipton, K.F. ed), vol. 26 pp.121-178. University Park Press, Baltimore.

Phillips, J.H., Burridge, K., Wilson, S.P., and Kirshner, N. (1983) J. Cell Biol. **97**:1906-1917.

Phillips, J.H., and Pryde, J.G. (1986) Annals New York Acad. Sci. (in the press).

Pletscher, A., Da Prada, M., Berneis, K.H., Steffen, H., Lutold, B. and Weber, H.G. (1974) In Advances in Cytopharmacology (eds. Ceccarelli, B., Clementi, F., and Meldolesi, J.), vol.2. pp.257-264.

Pollard, H.B., Miller, A. and Cox, G.C. (1973) J. Supramol. Struct. **1**:295-306.

Potter, V.R. (1967) J. Pharmac. Exp. Ther. **156**:500-506.

Pryde, J.G. (1982) M. Phil. Thesis University of Edinburgh.

Pryde, J.G. (1986) Trends Biochem. Sci. **11**:160-163.

Pryde, J.G., and Phillips, J.H. (1986) Biochem. J. **233**:525-533.

Pullman, M.E., Penefsky, H.S., Datta, A., and Racker, E. (1960) J. Biol. Chem. **235**:3322-3329.

Quinn, P., Griffiths, G., and Warren, G. (1983) J. Cell Biol. **96**:851-856.

Raff, M.C., and De Petris, S. (1973) Fed. Proced. **32**:48-54.

Rambourg, A., and Leblond, C.P. (1967) J. Cell Biol. **32**:27-53.

Reitman, M.L., Trowbridge, I.S. and Kornfeld, S. (1982) J. Biol. Chem. **257**:10357-10363.

Rindler, M.J., Ivanov, I.E., Rodriguez-Boulan, E., and Sabatini, D.D. (1982) In: Membrane Recycling, Ciba Foundation Symposium (D. Evered and G.M. Collins, eds.), vol.92 pp.184-208. (London:Putnam).

Role, L.W., and Perlman. R.L. (1980) J. Neuroscience Methods **2**:253-256.

Rome, L.H., Weissmann, B., and Neufeld, E.F. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**:2331-2334.

Rosa, P., Fumagalli, G., Zanini, A., and Huttner, W.B. (1985a) J. Cell Biol. 100:928-937.

Rosa, P., Hille, A., Lee, R.W.H., Zanini, A., De Camilli, P., and Huttner, W.B. (1985b) J. Cell Biol. 101:1999-2011.

Rosa, P., and Zanini, A. (1981) Mol. Cell Endocrinol. 24:181-193.

Rosa, P., and Zanini, A. (1983) Eur. J. Cell Biol. 31:94-98.

Roth, J. (1984) J. Cell Biol. 98:399-406.

Roth, J., and Berger, E.G. (1982) J. Cell Biol. 93:223-229.

Roth, J., Taatjes, D.J., Lucocq, J.M., Weinstein, J., and Paulson, J.C. (1985) Cell 43:287-295.

Rothman, J.E. (1981) Science (Washington) 213:1212-1219.

Rothman, J.E., Fries, E., Dunphy, W.G., and Urbani, L.J. (1982) Cold Spring Harbour Symp. Quant. Biol. 46:797-805.

Rothman, J.E., Miller, R.L., and Urbani, L.J. (1984a) J.Cell Biol. 99:260-271.

Rothman, J.E., Urbani, L.J., and Brands, R. (1984b) J. Biol. Chem. 99:248-259.

Sabatini, D.D., Kreibich, G., Morimoto, T., and Adesvik, M. (1982) J. Cell Biol. 92:1-22.

Sabban, E.L., and Goldstein, M. (1984) J. Neurochem. 43:1663-1668.

Sabban, E.L., Green, L.A. and Goldstein, M. (1983) J. Biol. Chem. 258:7812-7818.

Saraste, J., and Hedman, K. (1983) The EMBO J. 2:2001-2006.

Saraste, J., and Kuismanen, E. (1984) Cell 38:535-549.

Satre, M., Bof. M., Issartel, J-P., and Vignais, P.V. (1982) Biochemistry 21:4772-4776.

Saxena, A., and Fleming, P.J. (1983) J. Biol. Chem. 258:4147-4152.

Schachter, H., Jabbal, I., Hudgin, R.L., Pinteric, L., McGuire, E.J., and Roseman, S. (1970) J. Biol. Chem. 245:1090-1100.

Schachter, H., McGuire, E.J. and Roseman, S. (1971) J. Biol. Chem. 246:5321-5328.

Schachter, H., and Roseman, S. (1980) In: The Biochemistry of Glycoproteins and Proteoglycans. (W.J. Lennarz, ed.), pp.85-160. Plenum Press, New York.

Schlessinger, J., Axelrod, D., Koppel, D.E., Webb, W.W., and Elson, E.L. (1977) Science 195:307-309.

Schmidt, M.F.G., Bracha, M., and Schlesinger, M.J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:1687-1691.

Schmidt, M.F.G., and Schlesinger, M.J. (1980) J. Biol. Chem. 255:3334-3339.

Schneider, F.H. (1972) Biochem. Pharmacol. 21:2627-2634.

Schwarz, J.K., Capasso, J.M., and Hirschberg, C.B. (1984) J. Biol. Chem. 259:3554-3559.

Schwarz, R.T., Rohrschneider, J.M., and Schmidt, M.F.G. (1976) J. Viriol. 19:782-791.

Sharoni, Y., Eimerl, S., and Schramm, M. (1976) J. Cell Biol. 71:107-122.

Sharp, R.R., and Richards, E.P. (1977) Biochim. Biophys. Acta 497:14-28.

Sharp, R.R. and Sen, R. (1978) Biochim. Biophys. Acta. 538:155-163.

Sheares, B.T., and Carlson, D.M. (1983) J. Biol. Chem. 258:9893-9898.

Siegel, V., and Walter, P. (1985) J. Cell Biol. 100:1913-1921.

Silsand, T., and Flatmark, T. (1974) Biochim. Biophys. Acta 359:257-266.

Skotland, T., Ljones, T., (1980) Biochim. Biophys. Acta 630:30-35.

Skotland, T., Ljones, T., Flatmark, T., and Sletten, K. (1977) Biochem. Biophys. Res. Commun. 74:1483-1489.

Smith, C.E. (1980) J. Histochem. Cytochem. 28:16-26.

Snider, M.D. and Robbins, P.W. (1982) J. Biol. Chem. 257:6796-6801.

Snider, M.D. and Rogers, O.C. (1984) Cell 36:753-761.

Somogyi, P., Hodgson, A.J., De Potter, R.W., Fischer-Colbrie, R., Schober, M., Winkler, H., and Chubb, I.W. (1984) Brain Res. Rev. 8:193-230.

Spiro, M.J., and Ball, E.G. (1958) Fedn. Proc. Am. Soc. Exp. Biol. 17314-316.

Steiner, D.F., Docherty, K., and Carroll, R. (1984) J. Cellular Biochem. 24:121-130.

Strous, G.J.A.M., and Lodish, H.F. (1980) Cell 22:709-717.

Strous, G.J.A.M., Van Kerkhof, P., Willemsen, R., Geuze, H.J., and Berger, E.G. (1983a) J. Cell Biol. 97:723-727.

Strous, G.J.A.M., Willemsen, R., van Kerkhof, P., Slot, J.W., Geuze, H.J., and Lodish, H.F. (1983b) J. Cell Biol. 97:1815-1822.

Stuart, A.E., Hudspeth, A.J., and Hall. Z.W. (1974) Cell Tiss. Res. 153:55-61.

Suchard, S.J., Corcoran, J.J., Pressman, B.C. and Rubin, R.W. (1981) Cell Biol. Int. Rep. 5:953-962.

Sutton, R., and Apps, D.K. (1981) FEBS Lett. 130:103-106.

Tarentino, A.L., and Maley, F. (1974) J. Biol. Chem. 249:811-817.

Tarentino, A.L., Plummer, T.H., and Maley, F. (1974) J. Biol. Chem. 249:818-824.

Tartakoff, A.M. (1980) Int. Rev. Exp. Pathol. 22:227-251.

Tartakoff, A.M. (1983) Cell 32:1026-1028.

Tartakoff, A.M., and Vassalli, P. (1977) J. Exp. Med. 146:1332-1345.

Tartakoff, A.M., and Vassalli, P. (1983) J. Cell Biol 97:1243-1248.

Tejedor, F., and Ballesta, J.P.G. (1982) Anal. Biochem. 127:143-149.

Terland, O., and Flatmark, T. (1980) Biochim. Biophys. Acta 597:318-330.

Tooze. J., and Tooze, S.A. (1986) J. Cell Biol. 103:839-850.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:4350-4354.

Trifaró, J.M., Poisner, A.M., and Douglas, W.W.(1967) Biochem. Pharmacol. 16:2095-2100.

Trifaró, J.M., and Duerr, A.C. (1976) Biochim. Biophys. Acta 421:153-167.

Trifaró, J.M., and Dworkind, J. (1971) Molec. Pharmacol. 7:52-65.

Trifaró, J.M., Duerr, A.C., and Pinto, J.E.B. (1976) Molec. Pharmacol. 12:536-545.

Tulsiani, D.R.P., Hubbard, S.L., Robbins, P.W., and Touster, O. (1982) J. Biol. Chem. 257:3660-3668.

Unanue, E.R., Ungewickell, E., and Branton, D. (1981) Cell 26:439-446.

Varki, A., and Kornfeld, S. (1981) J. Biol. Chem. 256:9937-9943.

Viveros, O.H., Diliberto, E.J., Hazum, E., and Chang, K-J. (1979) Molec. Pharmacol. 16:1101-1108.

Von Euler, U.S. and Lishajko, F. (1961) Acta Physiol. Scand. 51:348-356.

Waechter, C.J., and Lennarz, W.J. (1976) Ann. Rev. Biochem. 45:95-112.

Wagner, R.R., and Cynkin, M.A. (1971) Biochem. Biophys. Res. Commun. 45:57-62.

Waheed, A., Pohlmann, R., Hasilik, A., and von Figura, K. (1981) J. Biol. Chem. 256:4150-4152.

Wallach, D.F.H., and Lin, P.S. (1973) Biochim. Biophys. Acta 300:211-254.

Wallach, D., and Schramm, M. (1971) Eur J. Biochem. 21:433-437.

Walter, P., and Blobel, G. (1981) J. Cell Biol. 91:557-561.

Walter, P., and Blobel, G. (1983a) Cell 34:525-533.

Walter, P., and Blobel, G. (1983b) Methods Enzymol. 96:84-93.

Walter, P., Ibrahimi, I., and Blobel, G. (1981) J. Cell Biol. 91:545-550.

Wang, F-F.C. and Hirs, C.H.W. (1977) J. Biol. Chem. 252:8358-8364.

Warren, G.B., Featherstone, C., Griffiths, G., and Burke, B. (1983) J. Cell Biol. 97:1623-1628.

Weber, A. and Winkler, H. (1981) Neuroscience 6:2269-2276.

Widnell, C.C. (1972) J. Cell Biol. 52:542-558.

Wiedenmann, B., and Franke, W.W. (1985) Cell 41:1017-1028.

Wildman, R.J., Dewair, M., and Matthaei, H. (1981) J. Neuroimmunol. 1:353-364.

Wilson, S.P., and Kirshner, N. (1976) J. Neurochem. 27:1289-1298.

Wilson, P., Sharkey, D., Haynes, N., Courtoy, P., and Farquhar, M.G. (1981) J. Cell Biol. 91(2,Pt.2):417a.

Wilson, S.P., and Viveros, O.H. (1981) Exp. Cell Res. 133:159-169.

Winkler, H. (1976) Neuroscience 1:65-80.

Winkler, H. (1977) Neuroscience 2:657-683.

Winkler, H., Apps, D.K., and Fischer-Colbrie, R. (1986) Neuroscience 18:261-290.

Winkler, H., and Carmichiel, S. (1982) In: The Secretory Process, vol.1 The Secretory Granule (Poisner, A.M. and Trifaro, J.M., eds.), pp.3-79. Elsevier Biomedical Press, Amsterdam.

Winkler, H., Falkensammer, G., Patzak, A., Fischer-Colbrie, R., Schober, M., and Weber, A. (1984) In: Regulation of Transmitter Function. Proc. 5th Meeting Eur. Soc. Neurochem. (Vizi, E.S. and Magyar, K. eds.) pp. 65-73.

Winkler, H., Hörtnagl, H., Hörtnagl, H., and Smith, A.D. (1970) Biochem. J. 118:303-310.

Winkler, H., Schneider, F.H., Rufener, C., Nakane, P.K., and Hörtnagl, H. (1974) In Advances in Cytopharmacology (eds. Ceccarelli, B., Clemente, F., and Meldolesi, J.) vol.2. pp127-139. Raven Press, New York.

Winkler, H., Hörtnagl, H., Schopf, J.A.L., and Hörtnagl, H. (1971) Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmacol. 271:193-203.

Winkler, H., Schopf, J.A.L., Hörtnagl, H., and Hörtnagl, H. (1972) Naunyn -Schmiedebergs Arch. Exp. Pathol. Pharmacol. 273:43-61.

Winkler, H., and Westhead, E. (1980) Neuroscience 5:1803-1823.

Wood, S.L., Apps, D.K., and Phillips, J.H. (1985) Biochem. Soc. Trans. 13:710-711.

Woods, J.W., Doriaux, M., and Farquhar, M.G. (1986) J. Cell Biol. 103:277-286.

Wu, G.E., Hozumi, N., and Mariazdo, A. (1983) Cell 33:77-83.

Yokota, S., and Fahimi, M.D. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:4970-4974.

Yonetani, T., and Ray, G.S. (1965) J. Biol. Chem. 240:3392-3398.

Yost, C.S., Hedgpeth, J., ans Lingappa, V.R. (1983) Cell 34:759-766.

Young, R.W. (1973) J. Cell Biol. 57:175-189.

Zeligs, J.D., and Wollman, S.H. (1979) J. of Ultrastruct. Res. 66:53-77.

Zinder, O., Hoffman, P.G., Bonner, W.M., and Pollard, H.B. (1978) Cell Tiss. Res. 188:153-170.

*Journal of Neurochemistry*
Raven Press, New York


# Glycoproteins of the Chromaffin Granule Membrane: Separation by Two-Dimensional Electrophoresis and Identification by Lectin Binding

*Fiona S. Gavine, *James G. Pryde, †David L. Deane, and *David K. Apps

**Department of Biochemistry, University of Edinburgh Medical School; and †MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh, U.K.*

**Abstract:** The proteins of highly purified chromaffin-granule membranes were separated by one- or two-dimensional electrophoresis, then transferred to nitrocellulose sheets; glycosylation was investigated by binding of several different radioiodinated lectins. Over 20 different glycosylated components were identified; comparison with mitochondrial and microsomal fractions suggested that most of the major glycoproteins are genuine components of the chromaffin granule membrane, rather than contaminants originating in other organelles. Two-dimensional electrophoresis revealed heterogeneity within several of the glycoproteins, and this is ascribed to differences in the state of glycosylation, on the basis of shifts in electrophoretic mobility produced by treatment with neuraminidase. Neuraminidase treatment of chromaffin granule membranes also enhances the binding of many lectins. The identities of the lectin-binding bands are discussed: neither cytochrome $b_{561}$ nor the $F_1$-like ATPase appears to be glycosylated. Chromogranin A, although a glycoprotein, does not bind any of the lectins tested, but a number of concanavalin-A binding proteins, as well as dopamine β-hydroxylase, are present in the chromaffin granule lysate. **Key Words:** Chromaffin — Membrane — Glycoprotein — Lectin — Blotting — Neuraminidase. **Gavine F. S. et al.** Glycoproteins of the chromaffin granule membrane: Separation by two-dimensional electrophoresis and identification by lectin binding. *J. Neurochem.* **43**, 1243–1252 (1984).

Chromaffin granules, the secretory granules of the adrenal medulla, store and release catecholamines, adenine nucleotides, and a number of polypeptides including dopamine β-hydroxylase, chromogranin A, and enkephalins. Relatively large quantities of these granules can be obtained in a highly purified state, and the chromaffin granule has therefore been extensively studied as a model secretory granule (Winkler and Carmichael, 1982). This approach is justified by the demonstration of numerous biochemical similarities between secretory granules from the adrenal medulla and other sources. For example, cytochrome $b_{561}$, a major component of chromaffin granule membranes, is also present in adrenergic neurones and in the dense serotonin-storing granules of platelets (Flatmark et al., 1971; Johnson and Scarpa, 1981). An electrogenic, inwardly directed $H^+$-translocating ATPase occurs in the membranes of chromaffin granules, as well as in the secretory granules of platelets (Wilkins and Salganicoff, 1981; Grunstein and Furuya, 1982), noradrenergic synapses (Toll and Howard, 1978), mast cells (Johnson et al., 1980), pancreatic β-cells (Hutton and Peshavaria, 1982), and the pituitary (Scherman et al., 1982); and chromogranin A is structurally related to a secretory protein from the parathyroid (Cohn et al., 1982).

The components of the chromaffin granule membrane have been investigated in some detail; as electrophoretic techniques have improved, the number of proteins ascribed to the granule membrane has increased, and Abbs and Phillips (1980) recognized 40–60 proteins. Even this figure may be an underestimate, as two-dimensional electrophoresis re-

Received October 6, 1983; revised March 15, 1984; accepted March 26, 1984.

Address correspondence and reprint requests to David K. Apps, Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, U.K.

*Abbreviations used:* Con A, Concanavalin A; DBA, Horse gram lectin; HEPES, N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; LCL, Lentil lectin; MES, 2-(*N*-Morpholino)ethane-sulphonic acid; PMSF, Phenylmethylsulphonyl fluoride; RCA-1 and RCA-2, Ricin 1 and 2, respectively; SBA, Soybean agglutinin; SDS, Sodium dodecyl sulphate; UEA, Gorse lectin; WGA, Wheat germ agglutinin.

solves many proteins that comigrate during separation through one dimension (Apps et al., 1980).

A number of studies of the glycoproteins of the chromaffin granule membrane have been published. Huber et al. (1979) used the periodate-Schiff method of staining electrophoretograms and affinity chromatography on lectin columns to isolate detergent-solubilized glycoproteins; by this means they detected five glycosylated components. Cahill and Morris (1979) used fluorescein-conjugated lectins to detect glycoproteins, after separation on sodium dodecyl sulphate (SDS)-polyacrylamide slab gels, and by this means detected about 12 components. Abbs and Phillips (1980) used both chemical and lectin labelling, and their results are in good agreement with those of Cahill and Morris (1979). The studies of Roda et al. (1980) revealed a smaller number of concanavalin A-binding proteins, many of them of low molecular weight.

The sensitivity and resolution of detection are improved if, following electrophoretic separation, glycoproteins are transferred ("blotted") from the polyacrylamide gel to a cellulose nitrate sheet, which is then decorated with radioiodinated lectin (Glass et al., 1981; Hawkes, 1982). We now report an investigation of lectin binding by chromaffin granule membrane proteins, in which we have used this technique in conjunction with two-dimensional electrophoretic separation.

## MATERIALS AND METHODS

### Preparations

Chromaffin granule membranes were isolated from fresh bovine adrenal glands (Apps and Schatz, 1979). Mitochondria were collected from the 0.3 *M*/1.8 *M* interface in the first ultracentrifugation step, lysed in 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer, and membranes purified by centrifugation through 1.0 *M* sucrose, then by isopycnic centrifugation on 0.5 *M*–1.4 *M* linear sucrose gradients (40,000 rpm, 15 h, Beckman SW 41 rotor).

Microsomal membranes were fractionated by methods developed from those of Trifaro and Duerr (1976) and Bretz et al. (1980). The postmitochondrial supernatant from adrenal medulla homogenate was centrifuged over 1.4 *M* sucrose (45,000 rpm, 60 min, Beckman 45 Ti rotor), and membranes from the 0.3 *M*/1.4 *M* sucrose interface made 1.4 *M* in sucrose. Aliquots of 15 ml were overlaid with the following sucrose solutions: 0.5 *M* (15 ml); 0.85 *M* (20 ml); 1.15 *M* (20 ml), then centrifuged (45,000 rpm, 60 min, Beckman 45 Ti rotor). Fractions from the 0.85 *M*/1.15 *M*/1.40 *M* sucrose interfaces corresponded approximately to the $GF_2$ and $GF_3$ fractions of Bretz et al. (1980); the former was enriched 8–10-fold (relative to crude microsomes), in the Golgi marker galactosyl transferase, whereas the latter was not significantly enriched, and appeared to derive mainly from the endoplasmic reticulum.

Galactosyl transferase (UDP galactose:2-acetoamido-2-deoxy-D-glucosyl glycopeptide galactosyltransferase, EC 2.4.1.38) was assayed with ovomucoid as acceptor, by an adaptation of published methods (Bretz and Staubli, 1977; Bretz et al., 1980). Lectins (Table 1; Sigma Chemical, or Vector Laboratories) were dissolved in 0.05 *M* sodium phosphate, pH 7.2, containing 0.15 *M* NaCl and the appropriate hapten monosaccharide (10 m*M*), and were radioiodinated by the two-phase procedure of Tejedor and Ballesta (1982). They were next purified by passage through a column of Biogel P6DG. The final specific activity was $2 \times 10^6$–$2 \times 10^7$ Bq/mg protein, depending on the lectin. $^{14}$C-Labelled standard proteins were prepared by the method of Dottavio-Martin and Ravel (1978). Protease-digested chromaffin granules were prepared by treatment of intact chromaffin granules with 0.1 mg/ml protease (chymotrypsin, papain, or pronase) in 0.3 *M* sucrose, 10 m*M* HEPES-NaOH, pH 7.0; after incubation for 15 h at 20°C, with gentle agitation, the granules were treated with 0.5 m*M* phenylmethylsulphonyl fluoride (PMSF), and purified as usual with 0.1 m*M* PMSF added to the isolation media; membranes were prepared by lysis of the purified protease-treated granules.

Neuraminidase-treated chromaffin granule membranes were prepared by resuspending purified granule membranes, at a concentration of 2 mg protein/ml, in 0.05 *M* 2-(*N*-morpholino)ethanesulphonic acid (MES)-NaOH, pH 5.5, containing 0.5 units/ml neuraminidase (Sigma type VIII) and 0.1 m*M* PMSF. The membranes were subjected to three cycles of freezing and thawing, then incubated for 15 h at 20°C, and washed by centrifugation and resuspension.

### Electrophoresis

One-dimensional electrophoresis was performed on polyacrylamide slab gels with exponentially increasing acrylamide concentrations (6–15 or 8–15%, wt/vol) with a constant acrylamide:bisacrylamide ratio of 37.5:1, using the buffer system of Laemmli (1970). Before one-dimensional electrophoresis, membrane proteins were precipitated with 10 volumes of cold 1:1 acetone/ethanol, and redissolved in sample buffer containing 5% wt/vol SDS and, where required, 5% vol/vol 2-mercaptoethanol. Fifty micrograms of membrane protein was applied to each track of the gel. Two-dimensional electrophoresis was performed essentially as described by O'Farrell (1975). The ampholytes used were Bio-Rad 3-10 and LKB 9-11, in the ratio 5:1, and the focussing gel contained 2% Nonidet P40. The protein sample was dissolved in 8 *M* urea containing 2% wt/vol octaethylene-glycol dodecyl ether ($C_{12}E_8$) and 2% ampholyte; then purified digitonin was added to a final concentration of 0.5% wt/vol. The sample was centrifuged to remove the white precipitate and the supernatant applied at the cathodic end of the focussing gel.

Electrophoretic transfer of proteins to cellulose nitrate sheets was performed as described by Towbin et al. (1979). The transfer buffer was 0.02 *M* $Na_2HPO_4$, 0.02 wt/vol SDS, 20% vol/vol methanol. The sheets were washed for 4 h in serum albumin (3% wt/vol, in 0.15 *M* NaCl, 20 m*M* Tris-Cl, pH 7.2) then in $^{125}$I-lectin ($10^4$ Bq/ml) for 2–15 h, then finally rinsed (6–15 h) in several changes of the same buffer and dried in air. Autoradiography was for 1–7 days on Agfa-Gevaert Curix RP-1 film. Control incubations were performed with the appropriate hapten sugar (10–100 m*M*) added with the lectin.

## RESULTS

### One-dimensional electrophoretic separation of chromaffin-granule membrane glycoproteins

Figure 1 shows Coomassie Blue-stained slab gels of chromaffin membrane proteins separated on two different polyacrylamide gradients. The bands are numbered as far as possible by the system of Abbs and Phillips (1980); these workers used different standard proteins to calibrate their gels, and also gels of different acrylamide concentration. The molecular weight/mobility plots of gradient gels are nonlinear, and our molecular weight estimates are not always in agreement, but many bands can be identified with reasonable certainty by their intensity of staining. Figure 2 shows an autoradiograph of a replica of a gel in which identically loaded tracks were decorated with three different $^{125}$I-lectins. Because of different changes in size that occur on drying acrylamide gels and cellulose nitrate sheets, the final autoradiograph cannot be superimposed on the original gel; seven $^{14}$C-labelled standard proteins (approximately 200 Bq of each) were therefore always included, so that lectin-binding bands could be identified by their apparent molecular weights.

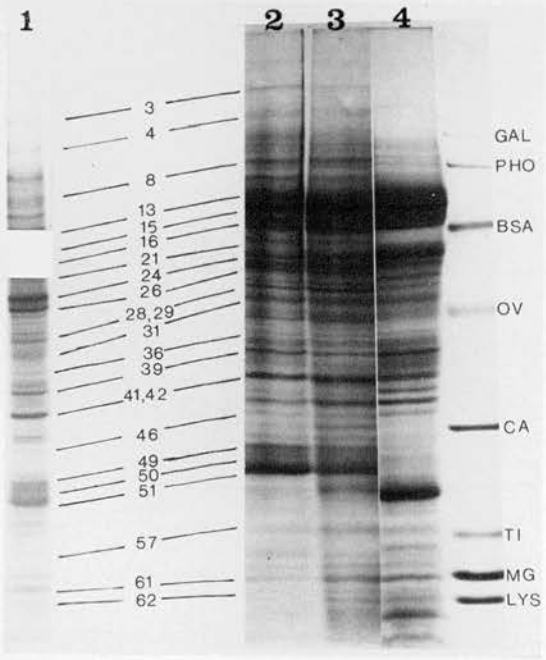



**FIG. 1.** One-dimensional electrophoretogram of chromaffin granule membranes, stained with Coomassie Blue. Acrylamide concentration 6–15% (track 1) or 8–15% (tracks 2–4). Tracks 1 and 2, untreated membranes; track 3, membranes treated after isolation with neuraminidase; track 4, membranes isolated from chymotrypsin-treated granules. Standard proteins: β-galactosidase (GAL, $M_r$ = 130,000); phosphorylase (PHO, 94,000); bovine serum albumin (BSA, 68,000); ovalbumin (OV, 43,000); carbonic anhydrase (CA, 30,000); trypsin inhibitor (TI, 21,500); myoglobin (MG,17,200); lysozyme (LYS, 14,300).

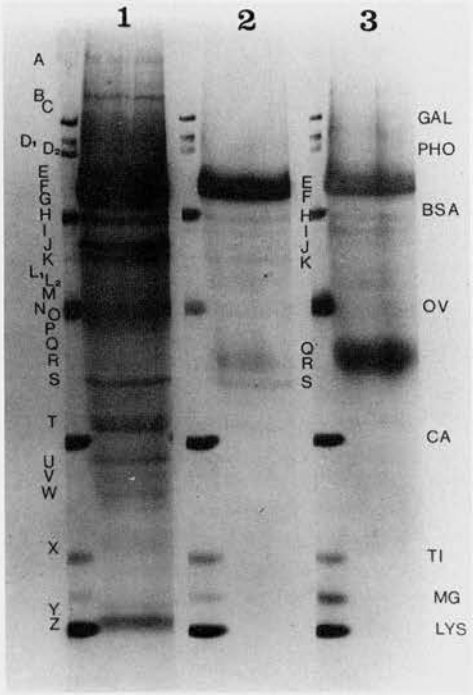



**FIG. 2.** Autoradiograph of chromaffin granule membrane proteins, separated by one-dimensional electrophoresis (acrylamide concentration, 8–15%) and labelled with $^{125}$-Con A (track 1), LCL (track 2), and WGA (track 3).

The greatest number of lectin-binding components is revealed by decoration with $^{125}$I-concanavalin A (Con A); these bands have been identified by letters (Fig. 2, track 1). Identical results were obtained using the method of Clegg (1982) in which Con A binding was revealed by peroxidase activity staining. It therefore appears that radioiodination does not affect the binding specificity of Con A. The binding of some other lectins is shown in Fig. 3. In this case the membranes had been pretreated with neuraminidase, to enhance the binding of these lectins (see below).

### Two-dimensional separation of glycoproteins

Several of the major membrane glycoproteins show microheterogeneity, and are poorly resolved by one-dimensional electrophoresis: these appear as diffuse bands or areas of high background staining in Figs. 1 and 2. Electrophoresis in two dimensions reveals these components much better, and Fig. 4a shows a two-dimensional electrophoretogram of chromaffin granule membrane proteins, stained with Coomassie Blue; Fig. 4b shows a similar gel, which was replicated on cellulose nitrate, decorated with $^{125}$I-Con A and autoradiographed. This procedure reveals seven major glycoproteins that are well resolved and therefore identifiable as sharp bands on one-dimensional electrophoresis;

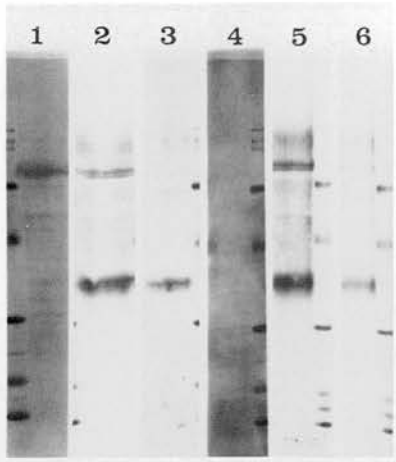



**FIG. 3.** Autoradiograph of neuraminidase-treated chromaffin-granule membrane proteins, separated by one-dimensional electrophoresis and labelled with $^{125}$I-DBA (track 1), RCA-2 (tracks 2 and 5), SBA (track 3), UEA (track 4), and RCA-1 (track 6).

these are bands B, E, F, G, H, J, and K. In addition, there are five components that show heterogeneity of molecular weight and isoelectric point, and therefore appear as diffuse areas. Two of these occur in the molecular weight range 100–130,000, and are overlaid by the minor components $D_1$ and $D_2$ (see Fig. 2); two have molecular weights 40–45,000 (the region occupied by bands N, O, and P) and one (band R), 36–40,000. R is labelled by a number of other lectins, including horse gram lectin (DBA), wheat germ agglutinin (WGA), ricin 1 (RCA-1), and ricin 2 (RCA-2).

Chromaffin granule membrane preparations are inevitably contaminated to some extent with proteins from the granule matrix (Apps et al., 1980), so it was important to establish whether any of the glycoproteins revealed by lectin binding were actually matrix components. A two-dimensional "map" of matrix proteins, decorated with $^{125}$I-Con A, shows a large number of minor components, in quite different positions from those of the membrane glycoproteins (Fig. 5). In this experiment, a narrow-range ampholyte (Biorad 4-6) was used for electrofocussing, as the chromaffin granule matrix proteins fall within a narrow range of isoelectric points, and are not particularly well separated on a wider pH gradient (Apps et al., 1980). Pictures of similar gels, stained with Coomassie Blue, have been published elsewhere (Kilpatrick et al., 1983).

### Correlation of Coomassie and lectin staining patterns

As discussed above, several of the glycoproteins are not readily detected by Coomassie staining; others occur in regions of the gel where several components comigrate. It is therefore difficult to correlate the protein staining pattern with lectin labelling, and we used a number of approaches to resolve this problem:

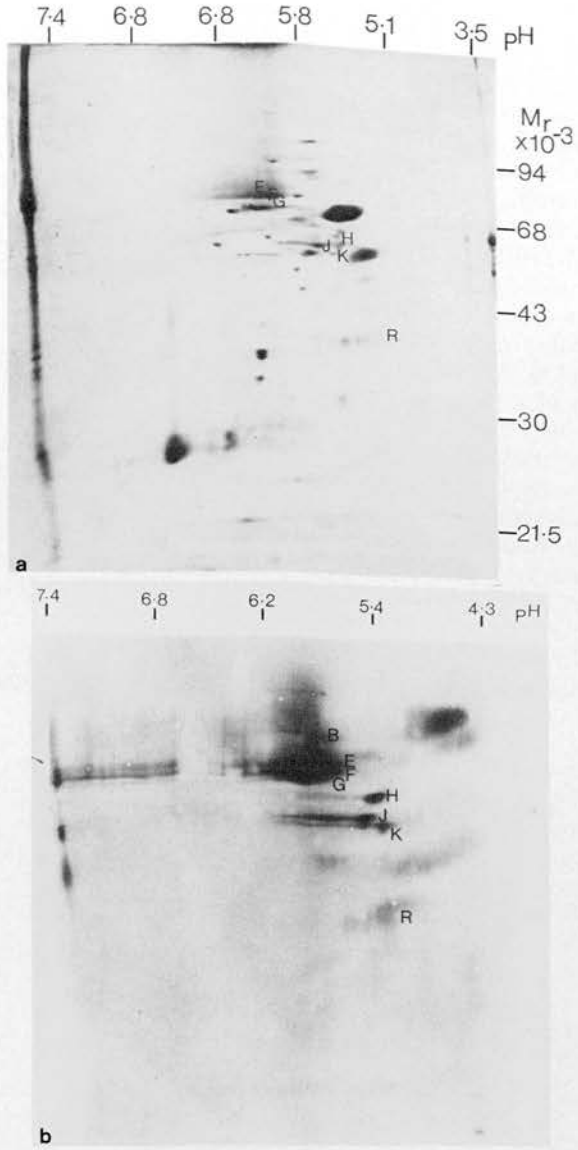



**FIG. 4. a:** Two-dimensional electrophoretogram of chromaffin granule membranes, stained with Coomassie Blue. **b:** Autoradiograph of chromaffin granule membrane proteins, separated by two dimensional electrophoresis and labelled with $^{125}$I-Con A. Letters refer to proteins identified in Fig. 2.

*Comparison of reduced and nonreduced protein samples.* The membrane proteins were routinely treated with 5% 2-mercaptoethanol before electrophoresis, to disaggregate polypeptide chains linked by disulphide bonds. Omission of mercaptoethanol results in significant changes in the Coomassie- and lectin-staining patterns, and the disappearance of certain bands from both allows their tentative identification as glycoproteins. The most obvious difference seen when the samples are not reduced with mercaptoethanol is the disappearance of the strong bands E and F, and a corresponding massive increase in the intensity of band A.

*Digestion with proteases.* Proteolysis of intact

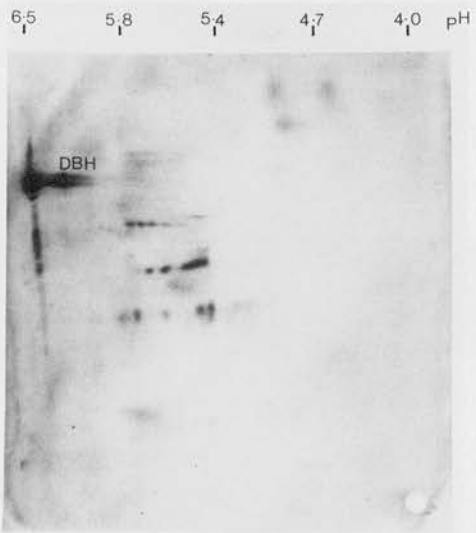



**FIG. 5.** Autoradiograph of chromaffin granule lysate proteins, separated by two-dimensional electrophoresis and labelled with $^{125}$I-Con A. For a similar gel stained with Coomassie Blue, see Kilpatrick et al. (1983).

chromaffin granules leads to partial degradation of many proteins that are exposed on the cytoplasmic face (Abbs and Phillips, 1980). When membranes were isolated from chromaffin granules that had been treated with chymotrypsin, papain, or pronase before purification, gel electrophoresis and Coomassie staining showed that several major membrane proteins had been degraded, the most prominent being cytochrome $b_{561}$ (Fig. 1). However, only a few changes appeared in the pattern of labelling by $^{125}$I-Con A (not shown); only transmembrane glycoproteins are affected by this treatment, since the carbohydrate moieties are known to be located on the matrix side of the membrane (Huber et al., 1979; Abbs and Phillips, 1980); alternatively, the presence of protease-sensitive bands could be due to contaminants. From such experiments it was possible to conclude that certain protease-sensitive Coomassie-stained bands were unlikely to be glycoproteins.

*Two-dimensional electrophoresis.* By the use of two-dimensional electrophoresis the positions of the major Coomassie Blue staining proteins can be matched with those of lectin-binding components, each protein being identified by its molecular weight, isoelectric point, and the shape of the spot.

### Neuraminidase treatment

The complex oligosaccharide chains of glycoproteins are frequently terminated with *N*-acetyl neuraminic acid; removing this with neuraminidase might be expected to enhance the binding of some lectins, by exposure of new terminal sugars. Several lectins [DBA, soybean agglutinin (SBA), and gorse lectin (UEA)] were indeed found not to label chromaffin granule membrane proteins at all, unless the membranes had been treated with neuraminidase before electrophoresis, whereas the binding of others (RCA-1 and RCA-2) was in general enhanced by neuraminidase treatment. Purified membranes may be vasiculated, and were therefore disrupted by freezing and thawing in the presence of the enzyme, before incubation with neuraminidase.

Neuraminidase treatment had no significant effect on the labelling of one-dimensional electrophoretic gels with Con A. However, two-dimensional electrophoresis revealed significant effects of neuraminidase treatment on the heterogeneous glycoprotein components discussed above in the section on two-dimensional separation of glycoproteins. In particular, band R (Fig. 6) appeared as a pattern of closely migrating components, the more acidic being of higher molecular weight. Some 10–12 components spread through a pH range of about 0.8 units, so the mean difference in pI is about 0.08; the molecular weight differences between these components are more difficult to estimate, as the spots have a greater vertical spread than others of comparable intensity, but the midpoints of the complete series of components span a molecular weight range of about 3,200. On treatment with neuraminidase, the proportion of the more basic, low-molecular-weight forms increased.

Neuraminidase treatment appeared to have some minor effects on the pattern of Coomassie Blue-

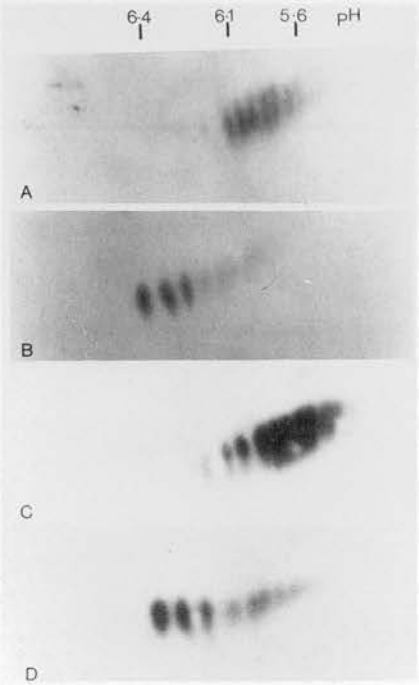



**FIG. 6.** Effects of neuramidase treatment on a heterogeneous glycoprotein of chromaffin granule membranes [band R, Fig. 3; glycoprotein III of Huber et al. (1979)]. After two-dimensional electrophoresis, labelling was with lentil lectin **(A, B)** or WGA **(C, D)**. (A, C), Untreated membranes; (B, D), neuraminidase-treated.

stained bands (Fig. 1), but similar changes in the Coomassie-stained bands were seen on overnight incubation of membrane samples without addition of neuraminidase, and therefore probably arose through the action of endogenous proteases (despite the inclusion of PMSF); the most noticeable change was a partial degradation of cytochrome $b_{561}$ (band 51), which is particularly susceptible to the actions of proteases. However, incubation of membranes without neuraminidase failed to produce any change in the lectin-binding pattern [e.g., of lentil lectin (LCL) or WGA to band R, or of SBA and UEA to other bands], and we therefore conclude that the neuraminidase-dependent changes in lectin binding were due to removal of sialic acid, rather than to the effect of proteases.

### Purity of chromaffin-granule membrane preparation

To assess the degree of contamination with membranes derived from other organelles, we prepared mitochondrial membranes and two microsomal fractions by sucrose density gradient centrifugation, for comparison of the distribution of Con A-labelled bands in these fractions with that in chromaffin-granule membranes (Fig. 7). This approach was not quantitative, as each subcellular fraction was contaminated with the others, and the intensity of bands in autoradiographs is not directly proportional to the amount of glycoprotein present, but depends on the efficiency of transfer of proteins to nitrocellulose, and on the state of glycosylation, which may be different in different fractions. Nonetheless it was possible to identify those bands that were present in highest concentration in Golgi fractions, and that therefore were probably not genuine constituents of the chromaffin granule membrane. Con A labelled no uniquely mitochondrial bands, but the use of other lectins revealed several glycoproteins that occurred only in the mitochondrial membrane fraction (not shown).

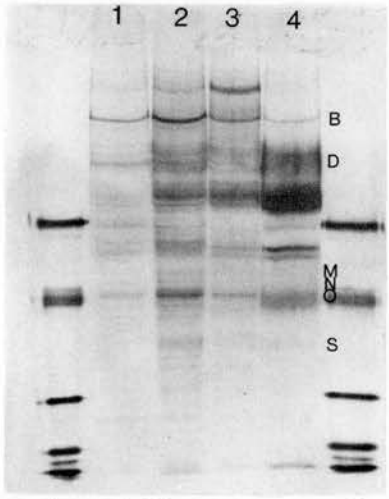



**FIG. 7.** Comparison of different membrane fractions from adrenal medulla, after labelling with $^{125}$I-Con A. Track 1, mitochondrial membranes; track 2, microsomes (1.15 *M*/1.40 *M* sucrose interface; see Materials and Methods); track 3, microsomes (0.85 *M*/1.15 *M* sucrose interface); track 4, chromaffin granule membranes.

## DISCUSSION

Table 1 lists the lectins used to decorate the gel replicas, and their sugar specificities. The results of one-dimensional electrophoretic separations, followed by binding of nine different lectins to the protein "blots," are summarized in Table 2. Where possible, we have identified the components with the numbers or letters used by other workers.

Are all the lectin-binding bands really glycoproteins? For a number of reasons we believe that they are: first, as observed by Cahill and Morris (1979), the binding of each lectin was specifically abolished by the hapten sugar; second, although many components bound more than one lectin, most lectins showed considerable selectivity; third, binding was very sensitive to the procedure used for iodinating the lectins, often not being observed if the standard chloramine-T procedure was used, or the protecting hapten sugar omitted; fourth, neuraminidase treatment of the membranes greatly increased the binding of several lectins, notably DBA, SBA, and UEA.

How many of the lectin-binding components are genuinely proteins of the chromaffin-granule membrane? We have no unequivocal way of assessing the level of contamination with such glycoprotein-rich fractions such as plasma membrane, Golgi, or endoplasmic reticulum (Apps et al., 1983), and mitochondrial membranes also contain components that bind Con A (Cidon and Nelson, 1982). However comparison of the labelling of different membrane fractions (Fig. 7) suggests that many of the components identified are present in chromaffin granules, being absent from the other fractions, or present only at lower levels: these are bands A, E, F, G, H, J, K, $L_1$, $L_2$, Q, R, T, U, V, and W. Other bands (B, $D_1$, $D_2$, M, N, O, and S) appear to be contaminants by this criterion. The location of some other bands (C, P, X, Y, and Z) is uncertain, either because they are faint or because they appear in all fractions. We therefore confine discussion to the major glycoproteins listed in Table 2.

### Dopamine β-hydroxylase

This enzyme [bands 13–15 in Abbs' and Phillips' (1980) nomenclature] comprises some 25% of the membrane protein. It contains about 4% carbohydrate, including mannose, galactose, fucose, *N*-acetyl glucosamine, and *N*-acetyl galactosamine (Fischer-Colbrie et al., 1982); as expected, it binds most of the lectins used in these studies. On Coomassie Blue-stained two-dimensional electropho-

**TABLE 1.** *Sources and specificities of the lectins used in these studies*

| Lectin | Source | Specificity |
|---|---|---|
| Concanavalin A (con A) | Jack bean (*Canavalia ensiformis*) | α-D-Man > α-D-Glc > α-D-GlcNAc |
| Horse gram lectin (DBA) | *Dolichos bifloros* | α-D-GalNAc |
| Lentil lectin (LCL) | *Lens culinaris* | α-D-Man > α-D-Glc > α-D-GlcNac |
| Peanut agglutinin (PNA) | *Arachis hypogaea* | β-D-Gal |
| Ricin 1 (RCA-1) | Castor bean | β-D-Gal > α-D-Gal |
| Ricin 2 (RCA-2) | (*Ricinis communis*) | α-D-GalNAc |
| Soybean agglutinin (SBA) | *Glycine max* | α-D-GalNAc > β-D-GalNAc |
| Gorse lectin (UEA) | *Ulex europaeius* | α-L-fucose |
| Weat germ agglutinin (WGA) | *Triticum vulgaris* | β-D-GlcNAc |

Man, Mannose; Glc, glucose; GlcNAc, *N*-acetyl glucosamine; Gal, galactose; GalNAc, *N*-acetyl galactosamine.

retograms, dopamine β-hydroxylase appears as a spot that is poorly focussed, and that has components of similar pI above and below it (Apps et al., 1980; Corcoran et al., 1982; Bader and Aunis, 1983); the upper two components bind mannose-specific lectins very strongly, so that dopamine β-hydroxylase appears as two bands (E and F) of similar intensity on autoradiographs of gel replicas decorated with Con A or LCL. It has been suggested (Skotland et al., 1977) that the enzyme has two types of subunit, which differ only in the three N-terminal amino acids, but this view has recently been questioned by Saxena and Fleming (1983), who concluded that the enzyme has two types of subunit of MW 70,000 and 75,000. We suggest that band F is the subunit usually visualized by Coomassie staining and that band E, with MW about 4,000 higher than that of F, is a derivative of higher carbohydrate content. Band G, which has the same pI as E and F, could be a degradation product, a derivative of lower carbohydrate content (accounting for its different lectin-binding properties) or even an entirely different protein. However, all three bands crossreact with antiserum raised against purified soluble dopamine β-hydroxylase.

Bands E and F are seen only when the membrane

**TABLE 2.** *Properties of chromaffin granule membrane glycoproteins*

| Band | $10^{-3}$ × Apparent MW | Con A | DBA | LCL | PNA | RCA-1 | RCA-2 | SBA | UEA | WGA | Identity (1) | (2) | (3) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 200 | + | | | | | | | | | 3A | I | 1 |
| B[a] | 150 | + + | | + | | | | | | + | 4 | | 3 |
| C | 145 | + | | | | | | | | | | | |
| $D_1/D_2$[a] | 107/100 | + | + | + | | | | + | + | + | 7/9B | II | |
| E | 78 | + + + + | + + | + + + + | | + + + + | + + + + | + | + | + + + | 13 C | | 6 |
| F | 74 | + + + + | + + | + + + + | | + + + | + + + | + | | + + + | 15 | | |
| G | 70 | + + + | + | | + | | | | | | 16 D | | |
| H | 64 | + + + | + | + | | | + | | | + + | 18 | | 7 |
| I | 60 | | | + | | | | | | + + | 21 | | |
| J | 56 | + + + | | + | | | | | | | 24 F | IV | 9 |
| K | 53 | + + + | | + | | | | | | | 25 | | 11 |
| $L_1/L_2$ | 51/49 | | + + | | | | + | | + | + | G | | |
| M | 47 | + | + + | | | | | | | | 31 | | |
| N | 44.5 | + + | | | + | | | | | | 32 H | V | 13 |
| O | 43 | + + | | | | | | | | | 34 | | |
| P | 40.7 | + | | | | | | | | | 36 | | |
| Q | 38 | | | | | | | | + | + + | 39 I | | 15 |
| R | 37 | | + + | + + | | + + + + | + + + + | + + + | + | + + + + | 40 J | III | |
| $S_1/S_2$[a] | 35 | + + + | + + + | + + | | | | | | | 41/42K | | |
| T[a] | 30.7 | + + + | + + + | | + | | | | | | 46 L | | |
| U[a] | 27.8 | + + | + | | | | | | | | 49 M | | |
| V[a] | 26.5 | + | + | | | | | | | | 50 | | |
| W[a] | 25.7 | + | + | | | | | | | | 51 | | |
| X | 21.7 | + | + | + | | + | | | | | 57 | | 22 |
| Y | 16.8 | + | + + + | | | | | | | | 61 | | |
| Z | 15.3 | + + + | + | | | | | | | | 62 | | |

Band letters refer to Fig. 2; [a]indicates a protease-sensitive band. Identities refer to (1) Abbs and Phillips (1980), who used numbers for Coomassie Blue-staining bands, and letters for glycoproteins; (2) Huber et al. (1979); (3) Cahill and Morris (1979). Strengths of labelling by different lectins are on the scale + (detectable) to + + + + (very strong). In the case of the lectins DBA, SBA, and UEA, labelling was detected only with membranes that had been pretreated with neuraminidase.

sample is reduced with mercaptoethanol; with omission of mercaptoethanol, band A is greatly intensified. Band A is therefore the undissociated, dimeric form of dopamine β-hydroxylase.

### Chromograinins

The chromaffin granule matrix contains a series of acidic proteins collectively called chromogranins, which inevitably contaminate isolated chromaffin granule membranes. Although they are mannose-containing glycoproteins (Fischer-Colbrie et al., 1982; Kiang et al., 1982) the chromogranins do not bind Con A or LCL, presumably because the mannose residues are not accessible to the lectins. As shown in Fig. 5, Con-A binds to dopamine β-hydroxylase, and to a number of minor components in the chromaffin granule lysate. These minor components do not react with an antiserum directed against dopamine β-hydroxylase, and they are therefore unlikely to be degradation products of the enzyme, so although dopamine β-hydroxylase is the major Con A binding protein of the granule matrix, it is not the only such component, in contrast to some previous reports.

### $H^+$-translocating ATPase

Chromaffin granule membranes appear to contain an ATPase that is structurally very similar to $F_1$-ATPase of mitochondria (Apps and Schatz, 1979), although a second, quite distinct ATPase has also been reported (Cidon and Nelson, 1983; Apps et al., 1983). Mitochondrial $F_1$-ATPase has been reported to be a glycoprotein (Andreu et al., 1978), but we have been unable to detect binding of any lectin to purified adrenal mitochondrial $F_1$, or to the chromaffin granule $F_1$-like ATPase, which has been identifed as bands 28, 29, and 44 in Coomassie-stained gels (Abbs and Phillips, 1980).

### Cytochrome $b_{561}$

The abundant chromaffin granule membrane protein of apparent MW 22–30,000, which was originally termed chromomembrin B, is now known to be cytochrome $b_{561}$ (Apps et al., 1980); it comprises some 15–20% of the membrane protein. The major component of purified cytochrome $b_{561}$ corresponds to band 51 (Fig. 1), with an apparent MW of 26,000, but bands 49 and 50 appear to be related (Hunter et al., 1982), and moreover, two-dimensional electrophoresis reveals charge heterogeneity of the major species (Apps et al., 1984). Although this heterogeneity might be explained by the existence of different glycosylated states of this protein, we have not detected binding of any lectin to purified cytochrome $b_{561}$. We tentatively conclude that cytochrome $b_{561}$ is not glycosylated, and that the comigrating bands U, V, and W are different proteins.

### Other major membrane glycoproteins

Apart from the components discussed above, few of the proteins of the chromaffin granule membrane have been identified, and the functions of several major lectin-binding components remain obscure.

A component of MW 100,000, revealed by two-dimensional electrophoresis (Fig. 4), has been recognized by other workers [e.g., Huber et al. (1979), who termed it glycoprotein II], and suggested to be a transmembrane protein, because it can be proteolytically degraded in intact granules (Abbs and Phillips, 1980); it was too faint to be identified unequivocally in one-dimensional gels, although it comigrates with bands $D_1/D_2$ (Table 2).

A component of MW about 37,000 (termed R) binds Con A, LCL, and WGA. This is identical with glycoprotein III of Huber et al. (1979), which migrates much more slowly in the electrophoretic system used by these authors than in that used in the present work (R. Fischer-Colbrie and H. Winkler, personal communication). We have confirmed this, using an antiserum raised against glycoprotein III. Band Q, apparent on one-dimensional separation (Fig. 2), is not revealed by electrophoresis in two dimensions, and may therefore be an artefact, arising from the heterogeneity of band R. As noted above, treatment with neuraminidase shifts the distribution of the closely comigrating components of band R, generating more of the components at low molecular weight and higher isoelectric point (Fig. 6); however, prolonged digestion did not convert all of the components to a single form. The molecular weight difference between each component is about 300, consistent with removal of a single sialic acid residue.

The large number of glycoproteins revealed in these studies is due to the sensitivity and resolution of the "blotting" technique which, unlike other carbohydrate-staining methods, produces bands of a sharpness comparable to those seen in Coomassie-stained gels.

It is difficult to draw firm conclusions about the relative carbohydrate contents of the bands, because of differences in efficiencies of transfer and in the specific radioactivity of the lectins, and because some lectins of the same stated sugar specificity apparently react with different subsets of protein. For example, Con A binds to many more bands than does LCL, even though both lectins are mannose-specific; but LCL labels band R more strongly than does Con A. The specific radioactivities of these lectins were quite similar ($2.0 \times 10^7$ and $1.1 \times 10^7$ Bq/mg, for Con-A and LCL, respectively) and chromatography of detergent-solubilized membrane proteins on immobilized lectin columns (not shown) confirms the results of the "blotting" experiments; it therefore appears that the binding of LCL can be determined by the immediate environment of the mannose. This also appears to be true for the fucose-specific lectin UEA, the binding of which is dependent on neuraminidase treatment of the membranes. Since fucose is invariably a ter-

minal sugar, it cannot be simply exposed by removal of sialic acid (as may be the case with galactose or *N*-acetyl galactosamine), but may become available to UEA through a conformation change.

The use of two-dimensional electrophoresis has proved valuable in detecting glycoproteins that are poorly resolved on SDS-polyacrylamide gel electrophoresis, and in revealing the differences between closely related glycoproteins. These techniques should be particularly useful in defining the relationships between the glycoproteins of secretory vesicles and those of the endoplasmic reticulum, Golgi, and plasma membrane.

**Acknowledgments:** This work was supported by a grant from the Scottish Home and Health Department. We thank Dr. J. H. Phillips and Dr. B. B. Cohen for invaluable advice and discussion of the manuscript, and Dr. R. Fischer-Colbrie for his gift of antiserum to chromaffin granule glycoprotein III.

## REFERENCES

Abbs M. T. and Phillips J. H. (1980) Organization of the proteins of the chromaffin granule membrane. *Biochim. Biophys. Acta* **595**, 200–221.

Andreu J. M., Warth R., and Munoz E. (1978) Glycoprotein nature of energy-transducing ATPases. *FEBS Lett.* **86**, 1–5.

Apps D. K., and Schatz G. (1979) An adenosine triphosphatase isolated from chromaffin-granule membranes is closely similar to $F_1$-adenosine triphosphatase of mitochondria. *Eur. J. Biochem.* **100**, 411–419.

Apps D. K., Pryde J. G., and Phillips J. H. (1980) Cytochrome $b_{561}$ is identical with chromomembrin B, a major polypeptide of chromaffin granule membranes. *Neuroscience* **5**, 2279–2287.

Apps D. K., Pryde J. G., and Sutton R. (1983) Characterization of detergent-solubilized adenosine triphosphatase of chromaffin granule membranes. *Neuroscience* **9**, 687–700.

Apps D. K., Boisclair M. D., Gavine F. S., and Pettigrew G. W. (1984) Unusual redox behaviour of cytochrome $b_{561}$ from bovine chromaffin granule membranes. *Biochim. Biophys. Acta* **764**, 8–16.

Bader M.-F. and Aunis D. (1983) The 97-KD α-actinin-like protein in chromaffin granule membranes from adrenal medulla: evidence for localization on the cytoplasmic surface and for binding to actin filaments. *Neuroscience* **8**, 165–181.

Bretz R. and Staubli W. (1977) Detergent influence on rat liver galactosyltransferase activities towards different acceptors. *Eur. J. Biochem.* **77**, 181–192.

Bretz R., Bretz H., and Palade G. (1980) Distribution of terminal glycosyltransferases in hepatic Golgi fractions. *J. Cell Biol.* **84**, 87–101.

Cahill A. L. and Morris S. J. (1979) Soluble and membrane lectin-binding glycoproteins of the chromaffin granule. *J. Neurochem.* **32**, 855–867.

Cidon S. and Nelson N. (1982) Properties of a novel ATPase enzyme in chromaffin granules. *J. Bioenerg. Biomembr.* **14**, 499–512.

Cidon S. and Nelson N. (1983) A novel ATPase in the chromaffin granule membrane. *J. Biol. Chem.* **258**, 2892–2898.

Clegg J. C. S. (1982) Glycoprotein detection in nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: application to arenavirus and flavevirus proteins. *Anal. Biochem.* **127**, 389–394.

Cohn D. V., Zangerle R., Fisher-Colbrie R., Chu L. L. H., Elting J. J., Hamilton J. W., and Winkler H. (1982) Similarity of secretory protein I from parathyroid gland to chromogranin A from adrenal medulla. *Proc. Natl. Acad. Sci. USA* **79**, 6056–6059.

Corcoran J. J., Lattanzio F. A., Rubin R. W., and Pressman B. C. (1982) Subcellular fractions of the adrenal medulla. Comparison by two-dimensional polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* **707**, 226–235.

Dottavio-Martin D. and Ravel J. M. (1978) Radiolabelling of proteins by reductive alkylation with [$^{14}$C]-formaldehyde and sodium cyanoborohydride. *Anal. Biochem.* **87**, 562–565.

Fischer-Colbrie R., Schachinger M., Zangerle R., and Winkler H. (1982) Dopamine β-hydroxylase and other glycoproteins from the soluble content and the membranes of adrenal chromaffin granules: isolation and carbohydrate analysis. *J. Neurochem.* **38**, 725–732.

Flatmark T., Lagercrantz H., Terland O., Helle K. B., and Stjarne L. (1971) Electron carriers of the noradrenergic storage vesicles from bovine splenic nerves. *Biochim. Biophys. Acta* **245**, 249–252.

Glass W. F., Briggs R. C., and Hnilica L. S. (1981) Use of lectins for the detection of electrophoretically separated glycoproteins transferred to nitrocellulose sheets. *Anal. Biochem.* **115**, 219–224.

Grunstein S. and Furuya W. (1982) A $Mg^{2+}$-stimulated ATPase in platelet α-granule membranes: possible involvement in proton translocation. *Arch. Biochem. Biophys.* **218**, 502–512.

Hawkes R. (1982) Identification of concanavalin-A binding proteins after SDS-gel electrophoresis and protein blotting. *Anal. Biochem.* **123**, 143–146.

Huber E., König P., Schuler G., Aberer W., Plattner H., and Winkler H. (1979) Characterization and topography of the glycoproteins of adrenal chromaffin granules. *J. Neurochem.* **32**, 35–47.

Hunter A., Waldron K., and Apps D. K. (1982) Determination of the proportion of sealed vesicles in a preparation of chromaffin granule membrane 'ghosts'. *FEBS Lett.* **144**, 51–56.

Hutton J. C. and Peshavaria M. (1982) Proton-translocating $Mg^{2+}$-dependent ATPase in insulin-secretory granules. *Biochem. J.* **204**, 161–170.

Johnson R. G. and Scarpa A. (1981) The electron transport chain of electron dense granules of platelets. *J. Biol. Chem.* **256**, 11966–11969.

Johnson R. G., Carty S. E., Fingerhood B. J., and Scarpa A. (1980) The internal pH of mast cell granules. *FEBS Lett.* **120**, 75–79.

Kiang W. L., Krusius T., Finne J., Margolis R. V., and Margolis R. K. (1982) Glycoproteins and proteoglycans of the chromaffin granule matrix. *J. Biol. Chem.* **257**, 1651–1659.

Kilpatrick L., Gavine F., Apps D. K., and Phillips J. H. (1983) Biosynthetic relationship between the major matrix proteins of adrenal chromaffin granules. *FEBS Lett.* **164**, 383–388.

Laemmli U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

O'Farrell P. H. (1975) High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021.

Roda L. G., Slater E. P., and Hogue-Angeletti R. A. (1980) Glycoproteins and glycopeptides in the chromaffin granule membrane. *Neurochem. Res.* **5**, 1243–1250.

Saxena A. and Fleming P. J. (1983) Isolation and reconstitution of the membrane-bound form of dopamine β-hydroxylase. *J. Biol. Chem.* **258**, 4147–4152.

Scherman D., Nordmann J., and Henry J.-P. (1982) Evidence of an adenine 5′-triphosphate dependent proton translocase in bovine neurosecretory granule membrane. *Biochemistry* **21**, 687–694.

Skotland T., Ljones T., Flatmark T., and Sletten K. (1977). $NH_2$-terminal sequence of dopamine β-hydroxylase from bovine adrenal medulla. *Biochem. Biophys. Res. Commun.* **74**, 1483–1489.

Tejedor F. and Ballesta J. P. G. (1982) Iodination of biological samples without loss of functional activity. *Anal. Biochem.* **127**, 143–147.

Toll L. and Howard B. (1978) Role of Mg-ATPase and a pH-gradient in the storage of catecholamines in synaptic vesicles. *Biochemistry* **17**, 2517–2523.

Towbin H., Staehelin T., and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.

Trifaro J. M. and Duerr A. C. (1976) Isolation and characterization of a Golgi-rich fraction from the adrenal medulla. *Biochim. Biophys. Acta* **421**, 153–167.

Wilkins J. A. and Salganicoff L. (1981) Participation of a transmembrane proton gradient in 5-hydroxytryptamine transport by platelet dense granules and dense-granule ghosts. *Biochem. J.* **198**, 113–123.

Winkler H. and Carmichael S. W. (1982) The chromaffin granule, in *The Secretory Granule* (Poisner N. and Trifaro J., eds), pp. 1–79. Elsevier Biomedical Press, Amsterdam.

Biochem. J. (1985) 231, 557–564 (Printed in Great Britain)



# Isolation of ATPase I, the proton pump of chromaffin-granule membranes

Judith M. PERCY, James G. PRYDE and David K. APPS
Department of Biochemistry, University of Edinburgh Medical School, George Square, Edinburgh EH8 9XD, U.K.

Chromaffin-granule membranes contain two ATPases, which can be separated by $(NH_4)_2SO_4$ fractionation after solubilization with detergents, or by phase segregation in Triton X-114. ATPase I ($M_r$ 400000) is inhibited by trialkyltin, quercetin and alkylating agents, and hydrolyses both ATP and ITP. It contains up to five types of subunit, including a low-$M_r$ hydrophobic polypeptide that reacts with dicyclohexylcarbodi-imide; these subunits are unrelated to those of mitochondrial $F_1F_0$-ATPase, as judged by size and reaction with antibodies. ATPase II ($M_r$ 140000) is inhibited by vanadate, and is specific for ATP; it has not been extensively purified. Proton translocation by resealed chromaffin-granule 'ghosts', measured by uptake of methylamine or by quenching of the fluorescence of 9-amino-6-chloro-2-methoxyacridine, is supported by the hydrolysis of ATP or ITP, and inhibited by quercetin or alkylating agents, but not by vanadate. ATPase I must therefore be the proton translocator involved in the uptake of catecholamines and possibly of other components of the chromaffin-granule matrix, whereas ATPase II does not translocate protons.

## INTRODUCTION

The secretory granules of the adrenal medulla, known as chromaffin granules, store and secrete catecholamines, nucleotides and proteins. The mechanism by which the very large intragranular concentrations of catecholamines are accumulated and maintained is now well established (Njus *et al.*, 1981): an electrogenic $H^+$-translocating ATPase acidifies the granule matrix, creating an ATP-dependent transmembrane pH gradient ($\Delta pH$) and a membrane potential ($\Delta\psi$), both of which are used to drive catecholamine/proton exchange by a separate, electrogenic, amine carrier. A chemiosmotic mechanism may also apply to the uptake of nucleotides by the granules; this appears to be driven by $\Delta\psi$ alone (Weber & Winkler, 1981), although in experiments with resealed chromaffin-granule 'ghosts' it has not been possible to generate the large nucleotide concentration gradients that this model predicts (Grueninger *et al.*, 1983).

Considerable progress has already been made in the identification and isolation of the amine carrier (Gabizon *et al.*, 1982; Scherman & Henry, 1983), but the nucleotide carrier is yet to be characterized. The nature of the $H^+$-translocating ATPase is controversial. Its inhibitor-sensitivity (Apps *et al.*, 1980*c*), catalysis of ATP/$[^{32}P]P_i$ isotope exchange (Roisin & Henry, 1982) and failure to catalyse [$^{14}$C]ADP/ATP isotope exchange (Apps & Reid, 1977) suggest a mechanistic and possibly structural similarity to the $F_1F_0$-ATPase of mitochondria, an idea supported by reconstitution studies (Buckland *et al.*, 1979; Giraudat *et al.*, 1980) and by electron microscopy (Schmidt *et al.*, 1982). An ATPase closely similar to mitochondrial ATPase was indeed found to be associated with chromaffin-granule membranes (Apps & Schatz, 1979), but it has since been shown (Cidon & Nelson, 1983; Cidon *et al.*, 1983) that chromaffin-granule membranes contain $H^+$-translocating ATPase activity after removal of all polypeptides that react with antibodies to mitochondrial $F_1$-ATPase. Furthermore, a hydrophobic DCCD-reactive protein of low $M_r$ was isolated from chromaffin-granule membranes and also shown to be structurally quite distinct from the mitochondrial DCCD-reactive proteolipid (Sutton & Apps, 1981); it appeared to be part of an ATPase complex (Apps *et al.*, 1982).

More recently it has been shown that purified chromaffin-granule membrane preparations contain, apart from small amounts of mitochondrial $F_1$-ATPase, two ATPases with quite different properties (Apps *et al.*, 1983). After solubilization with non-ionic detergent one of these enzymes (termed ATPase I) behaves on gel filtration in the presence of detergent as a complex of apparent $M_r$ 400000, is inhibited by DCCD and trialkyltin, and contains the DCCD-reactive subunit ($M_r$ 7000). The other (ATPase II) has an apparent $M_r$ of 140000, and is strongly inhibited by vanadate. By using the differences in inhibitor-sensitivity of the two ATPases, we have developed procedures for separating them, which yield ATPase I in a fairly pure state; we now report a partial structural characterization of this enzyme, and experiments with resealed chromaffin-granule 'ghosts' that correlate the inhibitor-sensitivity and substrate-specificity of nucleoside triphosphate-dependent amine uptake with those of ATPase I.

## MATERIALS AND METHODS

### Chemicals

Octa(ethylene glycol) dodecyl ether ($C_{12}E_8$) was obtained from the Kouyoh Trading Co. (Tokyo, Japan). Triton X-114 was obtained from Fluka, and purified by the method of Bordier (1981). Tributyltin and sodium orthovanadate were supplied by BDH Chemicals. Efrapeptin was from Eli Lilly Co. Quercetin, 4-chloro-7-nitrobenzofurazan, ATP (vanadate-free) and ITP

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; $C_{12}E_8$, octa(ethylene glycol) dodecyl ether; DCCD, *NN'*-dicyclohexylcarbodi-imide.

were from Sigma Chemical Co. Phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase were from Boehringer. *NN'*-Dicyclohexyl[$^{14}$C]carbodi-imide ([$^{14}$C]DCCD) was from C.E.A., Gif-sur-Yvette, France. Cholic acid (BDH Chemicals) was recrystallized from aq. 70% (v/v) ethanol. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was a gift from Dr. R. Kraayenhof (Vrije Universiteit, Amsterdam, The Netherlands), and duramycin was a gift from Dr. O. Shotwell (Midwest Area Northern Regional Research Center, Peoria, IL, U.S.A.).

### Assays

Hydrolysis of ATP or ITP was measured at 37 °C by spectrophotometric measurement of NADH oxidation in a medium of the following composition: 2 mM-ATP (or 2 mM-ITP), 10 mM-$MgSO_4$, 1 mM-phosphoenolpyruvate, 0.2 mM-NADH, 50 mM-KCl, 50 mM-Hepes/KOH buffer, pH 7.0, lactate dehydrogenase (3.6 units/ml) and pyruvate kinase (3.0 units/ml). ATP-dependent uptake of 5-hydroxy[$^{14}$C]tryptamine or of [$^{14}$C]methylamine by resealed chromaffin-granule 'ghosts' was measured as described previously (Apps *et al.*, 1980*c*). Quenching of ACMA fluorescence was measured at 25 °C in a Perkin–Elmer 3000 fluorimeter, with excitation and emission wavelengths of 400 nm and 525 nm respectively. The assay medium contained 3 mM-ATP (or 3 mM-ITP). 3 mM-$MgSO_4$, 40 mM-KI, 0.3 M-sucrose, 10 mM-Hepes/NaOH buffer, pH 7.6, 0.5 $\mu$M-ACMA and a 'ghost' concentration of 100 $\mu$g of protein/ml. Protein was determined by the modified Folin–Lowry procedure (Hartree, 1972), with bovine serum albumin as standard.

### Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on slab gels containing exponential gradients of 8–15% acrylamide, with the buffer systems of Laemmli (1970). Before electrophoresis, lipid and detergent were removed by precipitation of proteins with 50 vol. of acetone/ethanol (1:1, v/v) at −15 °C; the samples were redissolved in buffer containing 5% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol. Electrophoretic transfer to cellulose nitrate sheets and detection of mitochondrial $F_1$-ATPase were as described previously (Apps *et al.*, 1983). Scanning of Coomassie Blue-stained gels was on a Joyce–Loebl Chromoscan 3 densitometer. Radiolabelling of ATPase I was with 20 $\mu$M-[$^{14}$C]DCCD (specific radioactivity 1.85 TBq/mol) in the presence of 10 mM-ATP, 1 mM-EDTA and 10 mM-Hepes/NaOH buffer, pH 7.0, for 16 h at 4 °C. After electrophoresis, gels were fixed in methanol/acetic acid/water (2:1:7, by vol.), washed in water, soaked in 1 M-sodium salicylate for 1 h, dried on to paper and autoradiographed at −70 °C for 14 days.

### Fractionation methods

Chromaffin-granule membranes and resealed 'ghosts' were prepared as previously described (Apps *et al.*, 1980*c*). Fractionation of membrane proteins with $(NH_4)_2SO_4$ was by a modification of the procedure described previously (Apps *et al.*, 1980*b*). Membranes were suspended at a concentration of 4 mg of protein/ml in 20 mM-Hepes/NaOH buffer, pH 7.0, containing 1 mM-dithiothreitol and 0.1 mM-EDTA; $C_{12}E_8$ [10% (w/v) solution] was added to a final concentration of 10 mg/ml, and sodium cholate [10% (w/v) solution, pH 7.0] to 5 mg/ml. One-quarter volume of a saturated $(NH_4)_2SO_4$ solution, pH 7.0 was then added, and the solution was centrifuged in 12 ml tubes at 40000 rev./min for 30 min in a Beckman SW41 rotor ($1.96 \times 10^5$ $g_{av.}$). The white floating cake was removed from the solution, resuspended in 0.8 M-$(NH_4)_2SO_4$ and re-centrifuged, and then finally resuspended in one-tenth of the original volume of 20 mM-Hepes/NaOH buffer, pH 7.0, containing 1 mM-dithiothreitol and 0.1 mM-EDTA. The insoluble and soluble fractions were assayed for ATPase activity.

Fractionation with Triton X-114 was by a modification of the procedure of Bordier (1981). To chromaffin-granule membranes (4 mg of protein/ml in 150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.6), Triton X-114 [10% (w/v) stock] was added to give a final concentration of 2% (w/v). After a few minutes at 0 °C, the solution was centrifuged at 25000 rev./min for 30 min at 4 °C in a Beckman SW50.1 rotor ($5.8 \times 10^4$ $g_{av.}$). The supernatant was decanted and kept at 0 °C. The white pellet (P1) was resuspended in 150 mM-NaCl/10 mM-Tris buffer containing 2% Triton X-114, and re-centrifuged. This washing procedure was repeated in the absence of detergent, and the pellet was finally resuspended in 150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.6.

The supernatant (S1) was divided into 1.6 ml portions, and each was layered over 1.4 ml of 0.25 M-sucrose in 0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6, containing 0.06% Triton X-114; the tubes were warmed at 30 °C for 3 min, then centrifuged for 5 min at 4000 rev./min in a bench centrifuge ($2 \times 10^3$ $g_{av.}$). The aqueous layer was removed and kept at 0 °C, and the sucrose 'cushion' was decanted from the red pellet (P2), which was resuspended in 0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6, and again centrifuged through a sucrose 'cushion'; finally it was resuspended in 0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6.

Triton X-114 was added to the S2 fraction to give a final concentration of 0.5% at 0 °C, and then the solution layered on to sucrose 'cushions', warmed to 30 °C and centrifuged as before. The aqueous supernatant was again removed and made 2% in Triton X-114, warmed at 30 °C over sucrose 'cushions' and centrifuged again. The final aqueous supernatant (S2) was removed from the sucrose 'cushion' and retained.

The P1, P2 and S2 fractions were assayed for ATPase activity.

## RESULTS

### Fractionation of detergent-solubilized membranes

We previously (Apps *et al.*, 1983) reported separation of two distinct ATPases from chromaffin-granule membranes, by solubilization with taurodeoxycholate or with the non-ionic detergent $C_{12}E_8$, followed by exclusion chromatography on Sephacryl S-300, or centrifugation through glycerol density gradients, in the presence of $C_{12}E_8$. We have now developed two fractionation procedures that can be performed more rapidly, and on a larger scale. These are described in detail in the Materials and methods section.

The first method is derived from the procedure used to purify cytochrome *b*-561 from chromaffin-granule membranes (Apps *et al.*, 1980*b*); after solubilization with $C_{12}E_8$ and addition of cholate, $(NH_4)_2SO_4$ (20% saturation) precipitates a white lipid-rich fraction, which floats to the surface on centrifugation. This contains the DCCD-

**Table 1. ATPase and ITPase activities of chromaffin-granule membrane protein fractions produced by $(NH_4)_2SO_4$ fractionation, or by fractionation with Triton X-114**

'Recovery' refers to the total ATPase activity in each fraction. —, Not tested. For experimental details see the text.

| Fraction | Recovery (%) | Specific activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | ATPase (nmol/min per mg) | | | ITPase (nmol/min per mg) | | |
| | | Control | +2 μM-Tributyltin | +2 μM-Vanadate | Control | +2 μM-Tributyltin | +2 μM-Vanadate |
| Membranes | 100 | 220 | 40 | 170 | 60 | 20 | 60 |
| $(NH_4)_2SO_4$-insoluble | 15 | 470 | 30 | 490 | 100 | 40 | 90 |
| $(NH_4)_2SO_4$-soluble | 76 | 240 | 220 | 100 | 100 | 160 | 90 |
| Triton X-114 P1 | 19 | 550 | 10 | 530 | 100 | 30 | 90 |
| Triton X-114 P2 | 18 | 200 | 190 | 70 | 50 | 90 | 50 |
| Triton X-114 S2 | 1 | 10 | — | — | — | — | — |

sensitive ATPase activity (ATPase I), whereas the vanadate-sensitive ATPase activity (ATPase II) remains in solution. A detergent/protein ratio of 2.5:1 was routinely used; this produced the highest ATPase activity in the precipitated fraction, at the cost of some contamination with unwanted proteins (such as cytochrome *b*-561) compared with fractionation with a detergent/protein ratio of 5:1.

The second procedure is based on the temperature-dependent phase separation of Triton X-114 (Bordier, 1981), which segregates membrane proteins according to their hydrophobicity. After solubilization of chromaffin-granule membranes with this detergent at 0 °C, a precipitate forms, which contains ATPase I; the supernatant contains ATPase II, which, however, separates in the detergent-rich phase on warming at 30 °C.

In each case, ATPase I appears in the most hydrophobic fraction of detergent-solubilized proteins, the specific activity being increased 2–3-fold relative to chromaffin-granule membranes (Table 1); this is not an accurate reflection of the purification of ATPase I, since the activities of the two major ATPases in these membranes may be differently affected by solubilization. As shown in Table 1, they can be distinguished by their characteristic sensitivities to tributyltin and vanadate.

### Polypeptide content of separate ATPase fractions

The polypeptide composition of each fraction was determined by one-dimensional polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, after removal of lipid and detergent by precipitation with acetone/ethanol (Fig. 1).

The ATPase I fraction from the $C_{12}E_8/(NH_4)_2SO_4$ procedure (track 5) contains six major bands, of apparent $M_r$ 140000, 70000, 57000, 41000, 33000 and 16000: these are designated bands 1–6 respectively. There are traces of other components, but these are eliminated if a higher concentration of detergent is employed during solubilization of the membranes, with a slight loss of ATPase activity. The soluble fraction (track 6), with ATPase II activity, contains the remainder of the chromaffin-granule membrane proteins.

The first precipitate (P1) in the Triton X-114 procedure, which has ATPase I activity, also contains bands 1–6, together with some others (track 2); this precipitation is thus less selective than that obtained with $C_{12}E_8$. ATPase II occurs in the detergent-rich fraction P2 (track 3), which is, however, dominated by dopamine β-hydroxylase ($M_r$ 75000) and cytochrome *b*-561 ($M_r$ 27000). The soluble phase S2 (track 4) contains the most hydrophilic proteins, including the matrix protein

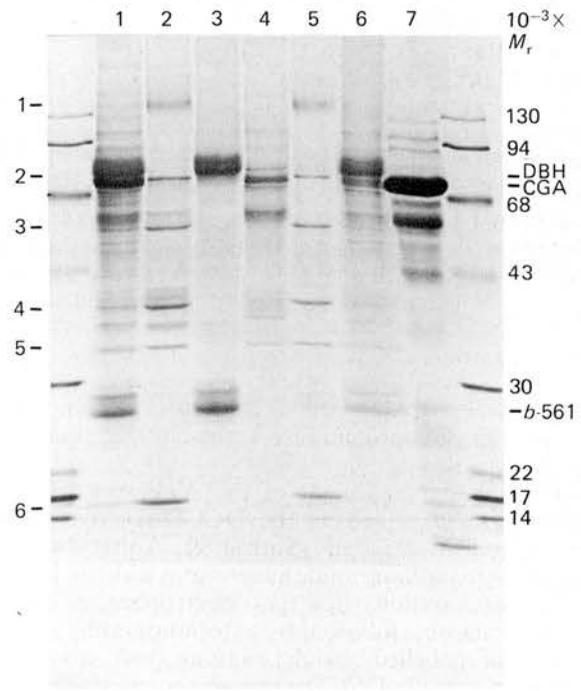



**Fig. 1. Gel electrophoresis of chromaffin-granule fractions**

Track 1, purified membranes; track 7, matrix proteins. Tracks 2, 3 and 4; Triton X-114 fractionation of membranes: P1, P2 and S2 fractions. Tracks 5 and 6, $(NH_4)_2SO_4$ fractionation of detergent-solubilized membrane proteins: insoluble and soluble fractions. Outer tracks, $M_r$ markers. Numbers to the left of the Figure refer to ATPase I polypeptides (tracks 2 and 5); the positions of dopamine β-hydroxylase (DBH, $M_r$ 75000, tracks 3 and 6), chromogranin A (CGA, $M_r$ 70000, tracks 4 and 7) and cytochrome *b*-561 (*b*-561, $M_r$ 27000, tracks 3 and 6) are also indicated.

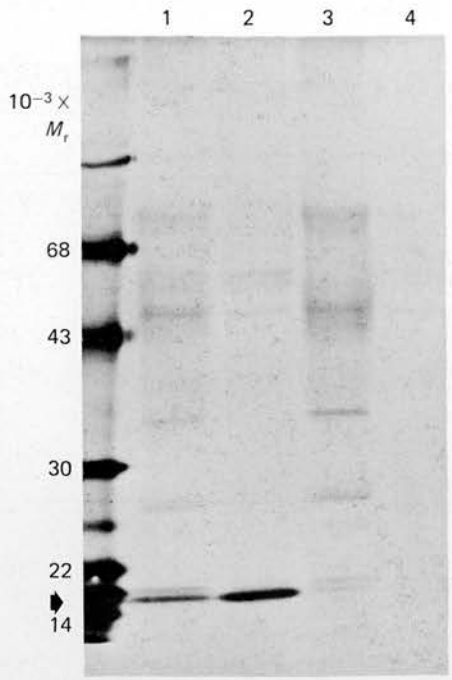



**Fig. 2. Autoradiograph of fractions obtained by Triton X-114 fractionation of membranes labelled with [$^{14}$C]DCCD**

Track 1, membranes; track 2, P1; track 3, P2; track 4, S2. Left-hand track, $^{14}$C-labelled $M_r$ markers. The position of the major chromaffin-granule DCCD-reactive protein (ATPase I, subunit 5) is indicated by a horizontal arrow.

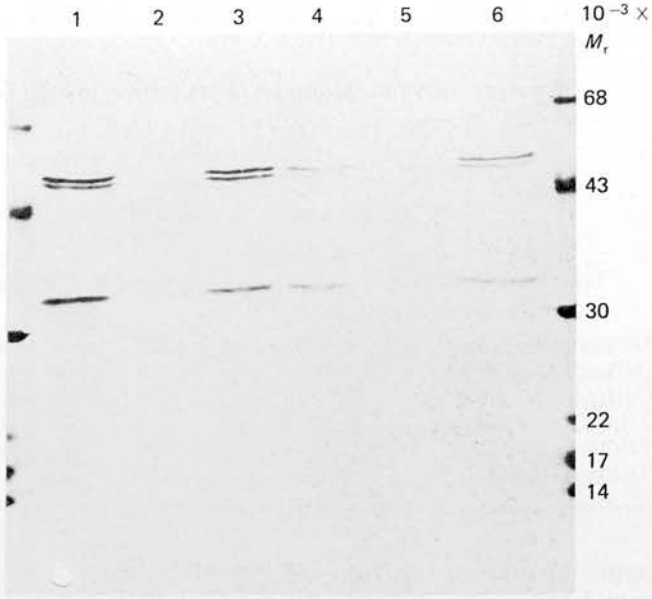



**Fig. 3. Distribution of mitochondrial $F_1$-ATPase during fractionation of chromaffin-granule membranes**

Proteins were separated by electrophoresis, transferred to a nitrocellulose sheet, decorated with anti-$F_1$-ATPase serum and $^{125}$I-protein A and autoradiographed. Track 1, membranes; tracks 2, 3 and 4, Triton X-114 fractionation of membranes: P1, P2 and S2 fractions. Tracks 5 and 6, $(NH_4)_2SO_4$ fractionation of detergent-solubilized membranes: insoluble and soluble fractions. Outer tracks, $^{14}$C-labelled $M_r$ markers.

chromogranin A ($M_r$ 70000) and several components identified as glycoproteins by lectin blotting (Gavine *et al.*, 1984).

The smallest component of the ATPase I fractions, band 6, was identified as the DCCD-reactive protein previously characterized (Sutton & Apps, 1981) by labelling chromaffin-granule membranes with [$^{14}$C]DCCD before fractionation, and gel electrophoresis of the separate fractions, followed by autoradiography (Fig. 2). The minor labelled band, running just above the chromaffin-granule DCCD-reactive protein, is the mitochondrial protein, which has a higher $M_r$ (Sutton & Apps, 1981) and is present here as a contaminant.

The distribution of mitochondrial $F_1$-ATPase was assayed by immune blotting (Towbin *et al.*, 1979), with an antiserum raised against bovine heart mitochondrial $F_1$, which reacts with the $\alpha$, $\beta$ and $\gamma$ subunits (Apps & Schatz, 1979). As shown in Fig. 3, only minute traces of this appear in the ATPase I fractions (tracks 2 and 5), most remaining in solution when ATPase I is precipitated.

### Use of inhibitors to distinguish ATPase I from ATPase II

In order to distinguish the two enzymes, we investigated the relative effects of single concentrations of several inhibitors on the various fractions (Table 2).

ATPase I is strongly inhibited by tributyltin, by quercetin and by preincubation with the alkylating agents *N*-ethylmaleimide and 4-chloro-7-nitrobenzofurazan; vanadate is not inhibitory. In contrast, ATPase II is very sensitive to vanadate, but only slightly affected by tributyltin, quercetin or the alkylating agents. Efrapeptin, a rather specific inhibitor of mitochondrial $F_1$-ATPase activity, does not inhibit ATPase I, although it has a slight inhibitory effect on the ATPase activity of the membranes, indicating some contamination by $F_1$-ATPase.

These results are in agreement with those obtained earlier with ATPases separated by exclusion chromatography (Apps *et al.*, 1983), apart from the effects of quercetin, which was previously found to inhibit the vanadate-sensitive ATPase. This question has now been investigated in more detail (see below).

### Hydrolysis of ITP by ATPase I

Chromaffin-granule membranes hydrolyse not only ATP, but also other nucleoside triphosphates; of these ITP is the best substrate (Kirshner, 1962). A comparison of the effects of inhibitors on the ITPase activity of chromaffin-granule membranes, and of the two ATPase fractions, shows that this activity is strongly inhibited by tributyltin in membranes and in the ATPase I fraction, whether this is prepared by $(NH_4)_2SO_4$ precipitation or by Triton X-114 fractionation (Table 1). Vanadate does not significantly affect the ITPase activity of any fraction. This suggests that ATPase I hydrolyses ITP, but that ATPase II does not; the ITPase activity of the ATPase II fraction is ascribed to contamination by vanadate-insensitive ATPases such as mitochondrial $F_1$-ATPase. Surprisingly, tributyltin stimulates the $(NH_4)_2SO_4$-soluble ITPase activity, though not ATPase; the reason for this is not known.

**Table 2. Effects of inhibitors on various ATPase fractions**

For experimental details see the text. Activities are expressed relative to controls assayed without inhibitors. For the alkylating agents 4-chloro-7-nitrobenzofurazan (NbfCl) and *N*-ethylmaleimide, assays were performed after preincubation of the enzyme (approx. 1 mg/ml, in 20 mM-Hepes/NaOH buffer, pH 7.0) with the inhibitor for 5 min at 20 °C.

| | | Relative activities | | | | |
|---|---|---|---|---|---|---|
| | | | | | Triton X-114 | |
| Inhibitor | Concentration | Membranes | $(NH_4)_2SO_4$-insoluble | $(NH_4)_2SO_4$-soluble | P1 | P2 |
| Tributyltin | 2 μM | 18 | 2 | 91 | 2 | 87 |
| Vanadate | 2 μM | 78 | 91 | 40 | 98 | 34 |
| NbfCl | 25 μM | 16 | 16 | 96 | — | — |
| *N*-Ethylmaleimide | 25 μM | 29 | 24 | 94 | — | — |
| Quercetin | 30 μM | 62 | 36 | 82 | 20 | 78 |
| Efrapeptin | 2 μg/ml | 91 | 100 | 93 | — | — |

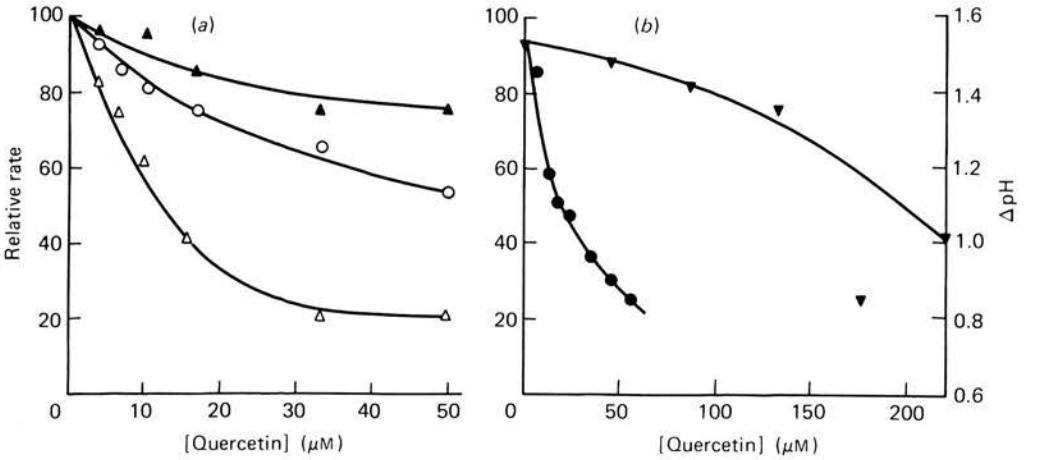



**Fig. 4. Effects of quercetin on ATPase and transport activities of chromaffin-granule membranes**

(*a*) ATPase activity of membranes (○), $(NH_4)_2SO_4$-insoluble fraction (ATPase I, △) and soluble fraction (ATPase II, ▲). (*b*) Resealed membrane 'ghosts': initial rate of uptake of 5-hydroxy[$^{14}$C]tryptamine (●) and steady-state ΔpH (▼).

### Inhibition by quercetin of $H^+$ translocation and amine uptake in resealed chromaffin-granule 'ghosts'

As shown in Table 2, quercetin is a relatively specific inhibitor of ATPase I activity, and is therefore useful for investigating the role of ATPase I in amine transport. Fig. 4(*a*) shows the effects of increasing concentrations of quercetin on the ATPase activity of chromaffin-granule membranes, and on separated ATPase I and II fractions, and Fig. 4(*b*) shows its effect on active transport in resealed chromaffin-granule 'ghosts', namely uptake of 5-hydroxy[$^{14}$C]tryptamine (which occurs via the catecholamine translocator) and generation of a transmembrane ΔpH (measured by the steady-state accumulation of [$^{14}$C]methylamine). Direct comparison of Figs. 4(*a*) and (*b*) is difficult because of the low protein concentrations used in ATPase assays (0.02–0.04 mg/ml) compared with those in transport experiments (0.5 mg/ml). Furthermore measurements of Δ pH were performed in the presence of 40 mM-KI to minimize $\Delta\psi$ and maximize ΔpH; this was necessary because methylamine distribution cannot accurately measure ΔpH values less than 1.0. The data in Fig. 4(*b*) establish that quercetin inhibits both ATP-dependent uptake of 5-hydroxytryptamine and $H^+$ translocation, but cannot be used to assess the variation of transport rates with ΔpH.

The rate of uptake of 5-hydroxytryptamine in pH-jump experiments (Apps *et al.*, 1980*a*) was unaffected by quercetin up to 100 μM (results not shown); this indicates that quercetin does not inhibit the catecholamine translocator, nor does it act as an uncoupler, collapsing the transmembrane ΔpH.

### $H^+$ translocation driven by hydrolysis of ATP

Hydrolysis of ITP supports only very low rates of uptake of 5-hydroxytryptamine (results not shown); this is consistent with the rather low ITP-induced steady-state ΔpH of 0.9, measured by the methylamine distribution technique. $H^+$ translocation into 'ghosts' can be investigated qualitatively by measuring quenching of the fluorescence of ACMA, a permeant weak base. As shown in Fig. 5(*a*), ATP produces a rapid quenching of ACMA fluorescence, which is reversed by the uncoupler carbonyl

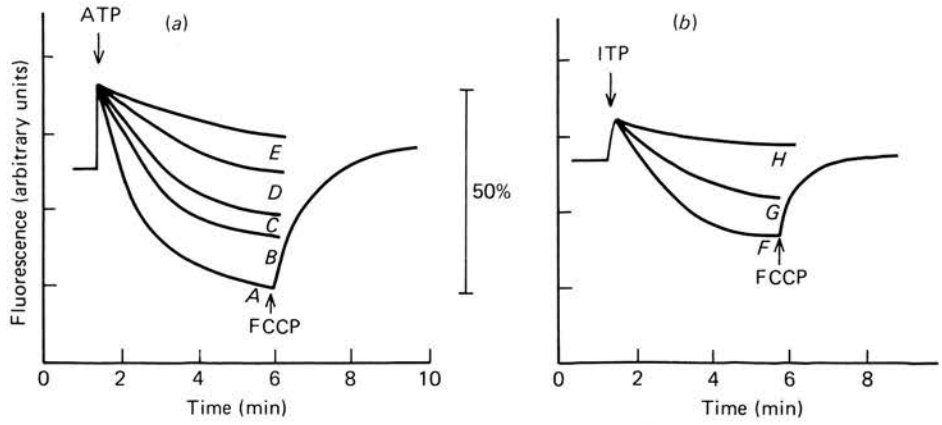



**Fig. 5. Quenching of ACMA fluorescence by resealed chromaffin-granule 'ghosts', induced by ATP (*a*) or ITP (*b*)**

'Ghosts' were preincubated with *N*-ethylmaleimide at various concentrations: 0 (traces *A* and *F*), 2.5 $\mu$M (trace *B*), 5 $\mu$M (trace *G*), 10 $\mu$M (traces *C* and *H*), 15 $\mu$M (trace *D*) or 20 $\mu$M (trace *E*). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to a final concentration of 5 $\mu$M (traces *A* and *F*). Duramycin (5 $\mu$g/ml) or vanadate (2 $\mu$M) had no effect on the ATP-dependent or ITP-dependent quenching, traces in presence of these inhibitors being superimposable on traces *A* and *F*.

cyanide *p*-trifluoromethoxyphenylhydrazone; preincubation of the 'ghosts' with *N*-ethylmaleimide inhibits fluorescence quenching, but vanadate is without effect. ITP produces a slower and less extensive quenching of ACMA fluorescence (Fig. 5*b*); like ATP-dependent quenching, this is inhibited by *N*-ethylmaleimide but not by vanadate. Duramycin, an inhibitor of proton pumping in clathrin-coated vesicles (Stone *et al.*, 1984), had no effect on acidification of 'ghosts' (as judged by the quenching of ACMA fluorescence) at a concentration of 5 $\mu$g/ml.

It is noticeable that ATP causes a small, non-energy-linked, quenching of ACMA fluorescence; this is seen in Fig. 5, which shows that addition of uncoupler does not produce a return of the fluorescence to exactly its starting value.

## DISCUSSION

The fractionation procedures described here separate the two major ATPases of the chromaffin-granule membrane; they can be carried out rapidly, and are easily applied on a fairly large scale. In each case ATPase I appears in the most hydrophobic fraction and is associated with a relatively small number of polypeptides, whereas the more soluble ATPase II is purified much less.

The first fraction in the $C_{12}E_8$/cholate/$(NH_4)_2SO_4$ procedure, which is discarded during the purification of cytochrome *b*-561 (Apps *et al.*, 1980*b*), contains ATPase I in the purest state yet achieved. Of the six bands apparent after one-dimensional electrophoresis (Fig. 1), the largest (band 1, approx. $M_r$ 140000) is an aggregate, since it has no equivalent in unfractionated membranes and is not seen if the sample is heated at 100 °C before electrophoresis (results not shown). The remaining five bands are all major components of the chromaffin-granule membrane, although band 2 ($M_r$ 70000) is obscured in one-dimensional electrophoretograms by dopamine $\beta$-hydroxylase and chromogranin A, and is only resolved from these by two-dimensional electrophoresis (results not shown). Band 6 is the DCCD-reactive protein described previously (Sutton & Apps, 1981); its apparent $M_r$ on polyacrylamide gels is 14000, but amino acid analysis and comparison with the mobility of the mitochondrial DCCD-reactive protein suggest an $M_r$ of 7000 (Apps *et al.*, 1982). If the chromaffin-granule membranes are contaminated with mitochondria, the mitochondrial DCCD-reactive protein, identifiable by its lower electrophoretic mobility, appears in the P2 fraction (J. G. Pryde, unpublished work).

It is not known which of these polypeptides (apart from band 6) are part of ATPase I. The same components are found in ATPase I purified by Triton X-114 fractionation (Fig. 1), exclusion chromatography (Apps *et al.*, 1983) or hydrophobic column chromatography (J. M. Percy, J. G. Pryde & D. K. Apps, unpublished work). The apparent $M_r$ of ATPase I is 400000 (Apps *et al.*, 1983), suggesting that the enzyme is a complex, but elucidation of its subunit stoichiometry must await further purification. Gel scanning indicated that ATPase I purified by different procedures contained different relative amounts of bands 1–6, suggesting either that some of these band proteins may not be part of the ATPase I complex, or that the extraction procedures cause some loss of subunits, thus accounting for the rather low recovery of activity. When one-dimensional polyacrylamide gels of chromaffin-granule membranes were scanned, only bands 3, 4 and 5 could be resolved from other membrane proteins; these are present in approximately equal amounts, equivalent to 200–250 copies per granule. A similar number of copies was calculated from band 6, by radiolabelling it with [$^{14}$C]DCCD (Apps *et al.*, 1983). The reliability of the scanning procedure was checked by scanning the cytochrome *b*-561 band; this was found to constitute about 20% of the membrane protein, in good agreement with the estimate of 19% obtained immunologically (Apps *et al.*, 1984).

We previously showed (Apps *et al.*, 1980*c*) that ATP hydrolysis, $H^+$ translocation and amine uptake by resealed chromaffin-granule 'ghosts' were inhibited by 4-chloro-7-nitrobenzofurazan, although this inhibitor had no effect on the catecholamine translocase itself, since amine uptake driven by an imposed pH gradient was not inhibited. Flatmark *et al.* (1982) found that another

alkylating agent, *N*-ethylmaleimide, completely blocked $H^+$ translocation at concentrations that only partially inhibited ATPase activity: even at high concentrations of *N*-ethylmaleimide, only 50% inhibition of ATPase activity occurred. Rather similar results have now been obtained with quercetin; this is a potent inhibitor of ATPase I, but has rather little effect on ATPase II; indeed, it is possible that the slight inhibition of this fraction by quercetin is due to contamination with ATPase I. Quercetin inhibits, not only ATPase activity, but also ATP-dependent uptake of 5-hydroxy[$^{14}$C]tryptamine (Fig. 4), yet it has no effect on the rate or extent of uptake of 5-hydroxytryptamine when this is driven by a rapid pH jump, which imposes a pH of about 2.1 (Apps *et al.*, 1980*a*). The relationship between ATP hydrolysis, pH generation and amine uptake is complex, since the steady-state concentration of catecholamines within the 'ghosts' depends on the square of the proton concentration ratio, or $2\Delta pH$ (Njus *et al.*, 1981), and $\Delta pH$ depends upon the rate of active $H^+$ translocation, passive $H^+$ leakage, internal buffering capacity and the concentration of permeant anions in the external medium. A relatively small change in $\Delta pH$ (Fig. 4*b*) may therefore have profound effects on the rate of uptake of 5-hydroxytryptamine.

Although ITP is a relatively poor substrate for the chromaffin-granule ATPase, ITP hydrolysis by 'ghosts' generates a significant $\Delta pH$, measurable by methylamine distribution or by quenching of ACMA fluorescence (Fig. 5). The inhibitor-sensitivity of ATPase activity (Table 1) suggests that ATPase I hydrolyses ITP but that ATPase II is more specific for ATP. Taken together, the substrate specificity of nucleoside triphosphate-driven $H^+$ translocation, and its sensitivity to quercetin, 4-chloro-7-nitrobenzofurazan and *N*-ethylmaleimide, show close parallels with the properties of ATPase I and demonstrate rather conclusively that ATPase I catalyses $H^+$ translocation. It seems unlikely that ATPase II translocates protons; quite apart from the redundancy of a second $H^+$ translocator in the same membrane, $\Delta pH$ generation and amine transport by 'ghosts' is completely insensitive to vanadate.

What structural and mechanistic similarities does ATPase I have to other $H^+$-translocating ATPases? Its high $M_r$ value suggests that it is a complex of several subunits, since the largest polypeptide in the ATPase I fraction has $M_r$ 70000. Inhibition by DCCD apparently occurs by covalent modification of the smallest subunit of the complex, a very hydrophobic protein that can be extracted into chloroform/methanol (Sutton & Apps, 1981). This presumably forms a $H^+$-conducting channel, since low concentrations of DCCD actually increase the ATP-generated $\Delta pH$ in 'ghosts' (Apps *et al.*, 1980*c*). The enzyme does not catalyse [$^{14}$C]ADP/ATP isotope exchange (Apps & Reid, 1977), suggesting that a phosphoryl-enzyme does not occur as an intermediate in the catalytic cycle; however, it does catalyse ATP/[$^{32}$P]$P_i$ isotope exchange, as would be expected for a reversible $H^+$-translocating ATPase (Roisin & Henry, 1982).

These properties clearly distinguish ATPase I from the $E_1E_2$ type of $H^+$-translocating ATPase, such as occurs in gastric mucosa and the fungal plasma membrane, and suggest some similarity to $H^+$-translocating ATPases of the $F_1F_0$ type, found in mitochondria, chloroplasts and bacteria. However, these ATPases are not inhibited by low concentrations of *N*-ethylmaleimide; the DCCD-reactive subunit of ATPase I shows significant differences from its mitochondrial counterpart; and, most significantly, antiserum raised against bovine mitochondrial $F_1$ does not react with any component of chromaffin-granule ATPase I (Fig. 3). It thus appears that, although ATPase I may be mechanistically similar to $F_1F_0$-ATPase, it is structurally similar only in its large size and in the fact that multiple subunits are attached to a DCCD-sensitive $H^+$-conducting channel. It is generally similar to the $H^+$-translocating ATPases that have been partially characterized in lysosomes (Moriyama *et al.*, 1984), Golgi vesicles (Glickman *et al.*, 1983), clathrin-coated vesicles (Xie *et al.*, 1984; Forgac & Cantley, 1984), the vacuolar membranes of fungi (Bowman & Bowman, 1982; Uchida *et al.*, 1985) and plants (Churchill & Sze, 1983), and the secretory vesicles of platelets (Dean *et al.*, 1984) and the pituitary (Russell, 1984). It may be that all of these enzymes are of the same type, and that work on chromaffin-granule ATPase I will yield results applicable to exocytotic and endocytotic organelles. However, it is noteworthy that chromaffin-granule ATPase I, unlike the $H^+$-translocating ATPase of coated vesicles, is not inhibited by duramycin.

It has been suggested that the knob-like protuberances seen on the cytoplasmic faces of chromaffin granules (Schmidt *et al.*, 1982) and synaptic vesicles (Stadler & Tsukita, 1984) may be the $H^+$-translocating ATPase. This is consistent with the proposed structure of ATPase I, and the diameter of these particles (8–9 nm) would be consistent with its $M_r$ value. However, at the measured frequency of 22 particles per granule, such particles would be only about 3% of the membrane protein, whereas bands 3–5 of the ATPase I fraction each comprise 2–3% of the membrane protein, or about 200 copies per granule. Although ATPase I may contain multiple copies of some subunits, these rough calculations suggest either that some of the bands in the ATPase I fraction are not part of the enzyme, or that the number of ATPase molecules per granule is underestimated by electron microscopy.

What is the significance of the $F_1$-like ATPase present in chromaffin-granule membrane preparations? Despite the fact that it is present in higher relative concentrations than mitochondrial markers (Apps *et al.*, 1983), it appears to be a contaminant: it is probable that $F_1$-ATPase particles become detached from mitochondria during cell fractionation, and associate with chromaffin granules to a greater extent than do intrinsic mitochondrial membrane proteins.

Little can be said of the structure or function of ATPase II. It has not been purified, and, since there is no specific assay for it, we cannot yet be certain that it is genuinely a component of chromaffin granules. After separation by exclusion chromatography (Apps *et al.*, 1983), ATPases I and II appear to have approximately equal activity, and to account for all of the ATPase activity of chromaffin-granule membranes; in the present study, the ATPase II activity recovered exceeds that of ATPase I, because ATPase I is partially inactivated by the fractionation procedure. The existence of two ATPases accounts for the partial inhibitions produced by several ATPase inhibitors when tested on intact membranes (Apps *et al.*, 1980*c*; Flatmark *et al.*, 1982). It is noteworthy that secretory vesicles from the pituitary also contain two ATPases, the properties of which are quite similar to those of the chromaffin-granule enzymes (Russell, 1984).

It is not clear which of the two enzymes was separated by Cidon & Nelson (1983), but it may be ATPase II, since it sedimented at a significantly lower rate than did mitochondrial $F_1$-ATPase; in their study, treatment with NaBr to remove $F_1$-ATPase apparently inactivated one of the chromaffin-granule ATPases as well.

This work was supported by a grant from the Medical Research Council. We thank Dr. J. H. Phillips for discussion of the manuscript.

## REFERENCES

Apps, D. K. & Reid, G. A. (1977) Biochem. J. **167**, 297–300
Apps, D. K. & Schatz, G. (1979) Eur. J. Biochem. **100**, 411–419
Apps, D. K., Pryde, J. G. & Phillips, J. H. (1980*a*) FEBS Lett. **111**, 386–390
Apps, D. K., Pryde, J. G. & Phillips, J. H. (1980*b*) Neuroscience **5**, 2279–2287
Apps, D. K., Pryde, J. G., Sutton, R. & Phillips, J. H. (1980*c*) Biochem J. **190**, 273–282
Apps, D. K., Pryde, J. G. & Sutton, R. (1982) Ann. N.Y. Acad. Sci. **402**, 134–145
Apps, D. K., Pryde, J. G. & Sutton, R. (1983) Neuroscience **9**, 687–700
Apps, D. K., Boisclair, M. D., Gavine, F. S. & Pettigrew, G. W. (1984) Biochim. Biophys. Acta **764**, 8–16
Bordier, C. (1981) J. Biol. Chem. **256**, 1604–1607
Bowman, E. J. & Bowman, B. J. (1982) J. Bacteriol. **151**, 1326–1337
Buckland, R. M., Radda, G. K. & Wakefield, L. M. (1979) FEBS Lett. **103**, 323–327
Churchill, K. A. & Sze, H. (1983) Plant Physiol. **71**, 610–617
Cidon, S. & Nelson, N. (1983) J. Biol. Chem. **258**, 2892–2898
Cidon, S., Ben-David, H. & Nelson, N. (1983) J. Biol. Chem. **258**, 11684–11688
Dean, G. E., Fishkes, H., Nelson, P. J. & Rudnick, G. (1984) J. Biol. Chem. **259**, 9569–9574
Flatmark, T., Gronberg, M., Husebye, E. & Berge, S. V. (1982) FEBS Lett. **149**, 71–74
Forgac, M. & Cantley, L. (1984) J. Biol. Chem. **259**, 8101–8105
Gabizon, R., Yetinson, T. & Schuldiner, S. (1982) J. Biol. Chem. **257**, 15145–15150
Gavine, F. S., Pryde, J. G. & Apps, D. K. (1984) J. Neurochem. **43**, 1243–1252
Giraudat, J., Roisin, M.-P. & Henry, J.-P. (1980) Biochemistry **19**, 4499–4505
Glickman, J., Croen, K., Kelly, S. & Al-Awqati, Q. (1983) J. Cell Biol. **97**, 1303–1308
Grueninger, H. A., Apps, D. K. & Phillips, J. H. (1983) Neuroscience **9**, 917–924
Hartree, E. F. (1972) Anal. Biochem. **48**, 422–427
Kirshner, N. (1962) J. Biol. Chem. **237**, 2311–2317
Laemmli, U. K. (1970) Nature (London) **227**, 680–688
Moriyama, Y., Takano, T. & Ohkuma, S. (1984) J. Biochem. (Tokyo) **95**, 995–1007
Njus, D., Knoth, J. & Zallakian, M. (1981) Curr. Top. Bioenerg. **11**, 107–147
Roisin, M. P. & Henry, J.-P. (1982) Biochim. Biophys. Acta **681**, 292–299
Russell, J. T. (1984) J. Biol. Chem. **259**, 9496–9507
Scherman, D. & Henry, J.-P. (1983) Biochemistry **22**, 2805–2810
Schmidt, W., Winkler, H. & Plattner, H. (1982) Eur. J. Cell Biol. **27**, 96–104
Stadler, H. & Tsukita, S. (1984) EMBO J. **3**, 3333–3337
Stone, D. K., Xie, X.-S. & Racker, E. (1984) J. Biol. Chem. **259**, 2701–2703
Sutton, R. & Apps, D. K. (1981) FEBS Lett. **130**, 103–106
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Uchida, E., Ohsumi, Y. & Anraku, Y. (1985) J. Biol. Chem. **260**, 1090–1095
Weber, A. & Winkler, H. (1981) Neuroscience **6**, 2269–2276
Xie, X.-S., Stone, D. K. & Racker, E. (1984) J. Biol. Chem. **259**, 11676–11678

Received 27 March 1985/24 June 1985; accepted 28 June 1985

Biochem. J. (1986) 233, 525–533 (Printed in Great Britain)



# Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114

## Application to subcellular fractions of the adrenal medulla

James G. PRYDE and John H. PHILLIPS
Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, U.K.

After solubilization with the detergent Triton X-114, membrane proteins may be separated into three groups: if the membrane is sufficiently lipid-rich, one family of hydrophobic constituents separates spontaneously at low temperature; warming at 30 °C leads to separation of a detergent-rich phase and an aqueous phase. Using the chromaffin-granule membrane as a model, we found that many intrinsic membrane glycoproteins are found in the latter phase, probably maintained in solution by adherent detergent. They precipitate, however, when this is removed by dialysis, leaving in solution those truly hydrophilic proteins that were originally adhering to the membranes. We have used this method with mitochondria, and with Golgi- and rough-endoplasmic-reticulum-enriched microsomal fractions: it has proved to be a rapid and convenient method for effecting a partial separation of proteins from a variety of different membranes.

## INTRODUCTION

There are several well-known problems associated with the analysis of membrane proteins: (1) in many cases it is difficult to obtain pure membrane fractions; (2) cytosolic, luminal or matrix proteins are inevitably associated with the membranes; and (3) no simple method exists to identify intrinsic proteins in the presence of such contamination. Electrophoretic analysis of membrane proteins has been improved by the introduction of two-dimensional separations, and the transfer of separated polypeptides to cellulose nitrate sheets has enhanced the sensitivity of antibody and lectin overlays. Using these techniques to analyse subcellular fractions of the adrenal medulla, we have developed some general methods which largely overcome these problems.

The methods were developed from the technique used by Bordier (1981) to separate mixtures of hydrophobic and hydrophilic proteins. The detergent Triton X-114, which forms clear micellar solutions in water at low temperatures, undergoes a phase separation owing to the formation of large micellar aggregates on warming above 20 °C. It is a good detergent for solubilizing membranes (Egan *et al.*, 1976) and this reversible phase-separation property can thus be used to separate intrinsic membrane proteins from the more hydrophilic proteins associated with membrane preparations. We report here that use of Triton X-114 allows the separation of up to four quite distinct families of proteins from purified membrane preparations. In particular, we have applied this method for a new analysis of the proteins associated with preparations of membranes of adrenal-medullary secretory granules (chromaffin granules).

Several detailed analyses of chromaffin-granule proteins have been published. Abbs & Phillips (1980), studying the topographical distribution of proteins by using one-dimensional electrophoresis and various protein-labelling techniques, showed that most of the Coomassie Blue-stained polypeptides were exposed to the outer (cytosolic) side of the secretory-granule membrane. They also confirmed the finding (Huber *et al.*, 1979) that the carbohydrate structures of membrane glycoproteins were on the inner or matrix face of the membrane. Subsequently more detailed electrophoretic analyses of the membrane and soluble matrix glycoproteins have been presented (Gavine *et al.*, 1984; Apps *et al.*, 1985). Techniques based on the use of Triton X-114 have enabled us to enhance the resolution obtained in previous studies, and seem also to be of wide applicability.

## MATERIALS AND METHODS

### Materials

Bovine adrenal glands were obtained from a local slaughterhouse and were placed on ice within about 20 min of slaughter. Triton X-114 was obtained from Fluka A.G., Buchs, Switzerland. Ampholytes were either from Bio-Rad Laboratories, Watford, Herts., U.K., or L.K.B., South Croydon, Surrey, U.K. Other electrophoresis materials were from B.D.H., Poole, Dorset, U.K.; cellulose nitrate sheets were from Schleicher and Schüll, Dassel, West Germany. A cholesterol assay kit was obtained from Boehringer Mannheim, Lewes, Sussex, U.K. Other biochemicals, including lectins, were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The lectins were iodinated as described by Gavine *et al.* (1984), to final specific radioactivities of $2 \times 10^6$–$2 \times 10^7$ Bq/mg of protein ($Na^{125}I$ was obtained from Amersham International, Amersham, Bucks., U.K.). *NN'*-Dicyclohexyl[$^{14}$C]carbodi-imide was obtained from C.E.A., Gif-sur-Yvette, France. The antiserum to mitochondrial $F_1$-ATPase from bovine heart was described by Apps & Schatz (1979). The pH of 1 M-Hepes solutions was adjusted with NaOH.

### Methods

**Membrane preparation.** Chromaffin granules were isolated from bovine adrenal glands in 0.3 M-sucrose containing 20 mM-Hepes, pH 7.0, and 0.2 mM-phenyl-

methanesulphonyl fluoride at 0 °C. After purification through buffered 1.8 M-sucrose, the contents were released by osmotic lysis and the membranes were purified over a 1.0 M-sucrose step gradient (Apps & Schatz, 1979). The membranes were washed and resuspended in 20 mM-Hepes, pH 7.0, containing 1 mM-dithiothreitol and 1 mM-EDTA, and were used either fresh or after storage at −20 °C. Mitochondrial membranes, and microsomal fractions enriched in the Golgi complex or rough endoplasmic reticulum, were prepared as described by Gavine *et al.* (1984). The lysate from the granules was supplemented with fresh 0.2 mM-phenylmethanesulphonyl fluoride and was dialysed against 1000 vol. of 1 mM-EDTA/1 mM-Hepes, pH 7.0. It was freeze-dried, dissolved in distilled water, centrifuged for 2 h at 150000 ***g*** at 4 °C, filtered through a 0.45 $\mu$m cellulose nitrate filter and stored at −20 °C.

The procedure of Howell & Palade (1982) was followed when membranes were washed with 0.2 M-$Na_2CO_3$.

**Triton X-114 treatment.** Triton X-114 was precondensed as described by Bordier (1981). It was used as a 10% (w/v) stock solution. Membranes were washed once in 0.15 M-NaCl/10 mM-Tris/HCl, pH 7.6 (Tris/salt buffer), then resuspended by homogenization. Triton X-114 was added at 0 °C, so that final concentrations were 2% (w/v) detergent and 4 mg of protein/ml. The solubilized membranes were placed on ice: after about 1 min a white precipitate formed. After a further 4 min, this was removed by centrifugation for 30 min at 58000 ***g*** in a Beckman SW50.1 rotor. The pellet was washed twice at 0 °C by resuspension to its original volume in Tris/salt buffer, first in the presence of 2% Triton X-114, and then in its absence.

The supernatant left after removal of this phospholipid-rich material was layered over a cushion of 0.25 M-sucrose in Tris/salt buffer (2 ml) containing 0.06% Triton X-114, in a conical glass centrifuge tube. After 5 min at 30 °C the solution became turbid. It was centrifuged for 5 min at 2500 ***g*** at room temperature in a swing-out rotor of a bench centrifuge. A detergent-rich phase collected under the cushion of sucrose.

The aqueous phase above the sucrose was removed and made 0.5% (w/v) with Triton X-114, mixed well and placed on ice for dissolution of the detergent. It was then replaced over the sucrose cushion used above, warmed at 30 °C for 5 min, and the tube was re-centrifuged. The resulting supernatant was cooled at 0 °C, made 2% (w/v) in Triton X-114, mixed well and then incubated for 5 min at 30 °C. It was centrifuged to remove the condensed detergent, which was discarded.

After washing of the centrifuge tube and the top of the sucrose cushion twice with Tris/salt buffer, the viscous detergent phase was diluted to its original volume with Tris/salt buffer and cooled to 0 °C to redissolve the detergent. This was warmed at 30 °C and the detergent-rich phase re-centrifuged through 0.25 M-sucrose. The resulting washed detergent-rich phase was collected, diluted with 1 or 2 vol. of Tris/salt buffer and stored at −20 °C.

**Dialysis of the aqueous phase.** Residual Triton X-114 (0.06%, as found by analysis) was removed from the aqueous phase by dialysis at 4 °C against Tris/salt buffer in which was dispersed 1% (w/v) Amberlite XAD-2. After 1–2 days the medium was replaced, and dialysis was continued. The dialysed fraction was then diluted with 3 vol. of water and centrifuged for 2 h at 165000 ***g***; this yielded a pellet which was washed and analysed separately from the supernatant, which was passed through a nitrocellulose filter (0.45 $\mu$m pore size). More than 90% of the Triton X-114 was removed by this procedure.

**Electrophoresis.** Samples for one-dimensional electrophoresis were delipidated in 10–75 vol. of acetone/ethanol (1:1, v/v) in silicone-treated Corex tubes in an ice/salt bath. They were then solubilized in sample buffer containing 5% (w/v) SDS and 5% (w/v) $\beta$-mercaptoethanol and analysed by electrophoresis in exponential (7–15 or 8–15%, w/v) polyacrylamide slab gels containing SDS as previously described (Gavine *et al.*, 1984). Isoelectric focusing for two-dimensional electrophoresis (O'Farrell, 1975) was on gels (11 cm × 0.3 cm) containing 4.5% (w/v) acrylamide, 0.06% (w/v) bisacrylamide, 9.5 M-urea, 2% (w/v) Triton X-100 and 2% (v/v) ampholytes (Bio-Rad 3–10 and LKB 9–11; 5:1, v/v). Samples were loaded at the basic (negative) end and the gels were focused for 30 min at 100 V, then at 200 V until the current was less than 0.5 mA per tube; the voltage was then increased to 400 V for 15–20 h, followed by 0.5 h at 800 V. Samples (200–300 $\mu$g of protein) were prepared by freeze-drying and solution in 9.5 M-urea containing 2% ampholytes, 2% Triton X-100, 5% $\beta$-mercaptoethanol and 15–30 $\mu$g of Bromophenol Blue/ml. They were clarified by centrifugation: any floating lipid was discarded. Samples in Tris/salt buffer were desalted by using small columns of Biogel P-6DG.

After electrophoresis, radioactive gels were fixed in methanol/acetic acid/water (2:1:7, by vol.) for 30 min, washed briefly in water, soaked in 1 M-sodium salicylate for 1 h, dried on Whatman 3 MM paper, then autoradiographed at −70 °C for 7–14 days.

Electrophoretic transfer of proteins to cellulose nitrate sheets and immunoblotting were performed by the method of Towbin *et al.* (1979), with the solutions previously described (Gavine *et al.*, 1984). After a wash in Tris/salt buffer containing 3% (w/v) bovine serum albumin for 60 min, the sheets were incubated in Tris/salt buffer containing 5% (v/v) inactivated horse serum and antiserum, usually diluted 100-fold. After five washes with Tris/salt buffer, they were treated with $^{125}$I-protein A (4 kBq/ml). After five further washes in Tris/salt buffer, one containing 0.1% Triton X-100, they were dried and autoradiographed for 1–7 days by using Agfa–Gevaert Curix RP-1 film.

**Decoration with lectins.** Cellulose nitrate replicas of gels were washed with periodate-treated bovine serum albumin (3%, in Tris/salt buffer) for 4 h. For this purpose 4% albumin in 0.1 M-acetic acid was treated with 10 mM-$NaIO_4$ for 6 h, at 20 °C. Glycerol was then added to 10 mM and the solution was dialysed against Tris/salt buffer containing $NaN_3$ (0.5 mg/ml).

The sheets were then washed five times in Tris/salt buffer and incubated overnight with $^{125}$I-labelled lectin (10 kBq/ml). They were then washed five more times, the penultimate wash containing 0.1% Triton X-100 (this was omitted when wheat-germ agglutinin had been used). Control replicas were incubated with $^{125}$I-lectin in the presence of the appropriate hapten sugar at 100 mM. Sheets were then autoradiographed for 3–21 days.

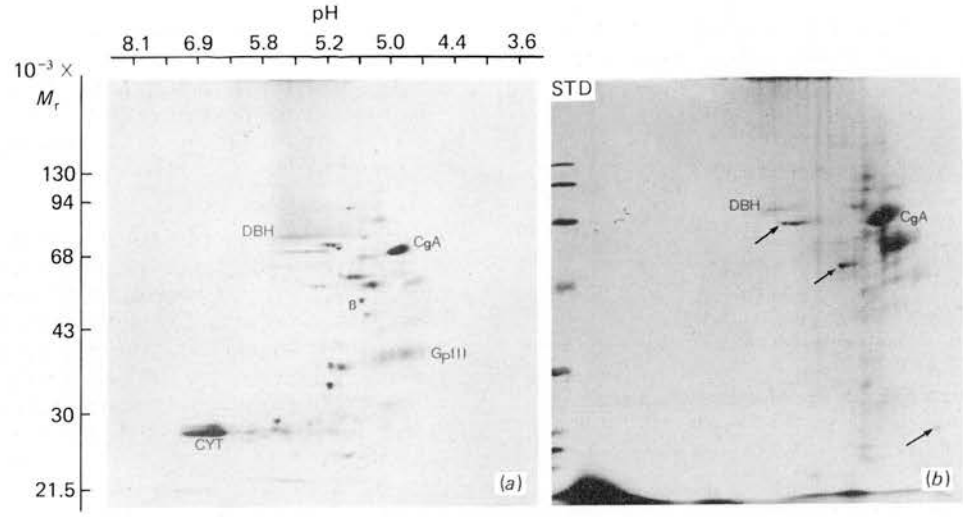



**Fig. 1. Two-dimensional electrophoretic analysis of chromaffin-granule membrane proteins**

(*a*) Complete membranes; (*b*) proteins removed by washing with $Na_2CO_3$. Arrows in (*b*) indicate proteins not found in the lysate of chromaffin granules, as identified from Fig. 1 of Apps *et al.* (1985). Abbreviations: DBH, dopamine β-hydroxylase; CgA, chromogranin A; β, β-subunit of $F_1$-ATPase; $G_P$III, glycoprotein III of Huber *et al.* (1979); CYT, cytochrome $b_{561}$; STD, standard proteins (one dimension): β-galactosidase ($M_r$ 130000); phosphorylase ($M_r$ 94000); bovine serum albumin ($M_r$ 68000); ovalbumin ($M_r$ 43000); carbonic anhydrase ($M_r$ 30000); trypsin inhibitor ($M_r$ 21500); myoglobin ($M_r$ 17200); lysozyme ($M_r$ 14300).

**Other methods.** Protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard. When detergent was present in samples, the method of Hartree (1972) was used. Lipid phosphorus determination was by a method based on that of Bartlett (1959). Triton was assayed by the method of Garewal (1973).

Chromaffin-granule membranes and mitochondrial membranes (4 mg of protein/ml) were covalently labelled with radioactive dicyclohexylcarbodi-imide by incubation in a solution containing 10 mM-Hepes, pH 7.4, 0.5 mM-EDTA, 10 mM-ATP and 20 μM-*NN'*-dicyclohexyl[$^{14}$C]-carbodi-imide (sp. radioactivity 1.85 GBq/mol). The samples were rotated slowly at 4 °C for 15 h, then washed in Tris/salt buffer.

Bovine heart $F_1$-ATPase, type I, was prepared by the method of Fisher *et al.* (1981).

## RESULTS

### Removal of adherent soluble proteins

The secretory granules of the adrenal medulla (chromaffin granules) are the best-characterized subcellular fraction of this tissue. Preparations of their membranes are, however, always contaminated by secretory proteins, in particular chromogranin A, as well as by cytosolic and extracellular components. Washing membranes with 0.2 M-$Na_2CO_3$ breaks open vesicular structures and removes extrinsic contaminants (Howell & Palade, 1982; Higgins, 1984).

A two-dimensional electrophoretic separation of chromaffin-granule membrane proteins is shown in Fig. 1(*a*). The major proteins of the membrane preparation are cytochrome $b_{561}$ ($M_r$ 26000, pI 6.2), dopamine β-hydroxylase ($M_r$ 75000, pI 5.4; this protein enters the second dimension poorly and is always revealed as a smear in the top part of the gel, and chromogranin A, the major matrix protein ($M_r$ 70000, pI 4.9). One of the major glycoproteins ($M_r$ 37000; glycoprotein III of Huber *et al.*, 1979) can also be seen. A distinct subset of proteins, almost identical with those released by lysis of intact granules (Apps *et al.*, 1985), is removed by the $Na_2CO_3$-washing procedure (Fig. 1*b*). These proteins have been characterized by immunological techniques and have been shown to contain chromogranins A and B, and the products of their proteolysis (Fischer-Colbrie & Frischenschlager, 1985), and a small amount of the matrix enzyme dopamine β-hydroxylase. There are, however, a few non-matrix proteins (shown in Fig. 1*b*), which are almost certainly adhering to the cytosolic surface of the granule membrane: among these are the β-subunit of mitochondrial $F_1$-ATPase ($M_r$ 50000, pI 5.1), and three components that we also find on gels of endoplasmic reticulum and Golgi fractions. After two or three cycles of washing, depletion of these components from the membrane preparation is almost complete; a trace of chromograin A remains as the most conspicuous contaminant. Having identified this set of proteins in this way, we did not normally prewash membranes before using the phase-separation procedure.

### Solubilization and temperature-induced phase separation with Triton X-114

Triton X-114, when used at a detergent:protein ratio of 5:1 at 0 °C, was a good agent for solubilizing chromaffin-granule membrane proteins. However, unlike solubilization with Triton X-100 or octaethyleneglycol dodecyl ether (Apps *et al.*, 1980), a white precipitate spontaneously separated from the resulting solution. This effect was not pH-dependent; the pellet was composed of aggregates of detergent, phospholipid and cholesterol, together with some very hydrophobic proteins (approx. 10% of the chromaffin-granule membrane protein). We refer to the pellet as the phospholipid-rich phase.

On warming at 30 °C, the supernatant became turbid

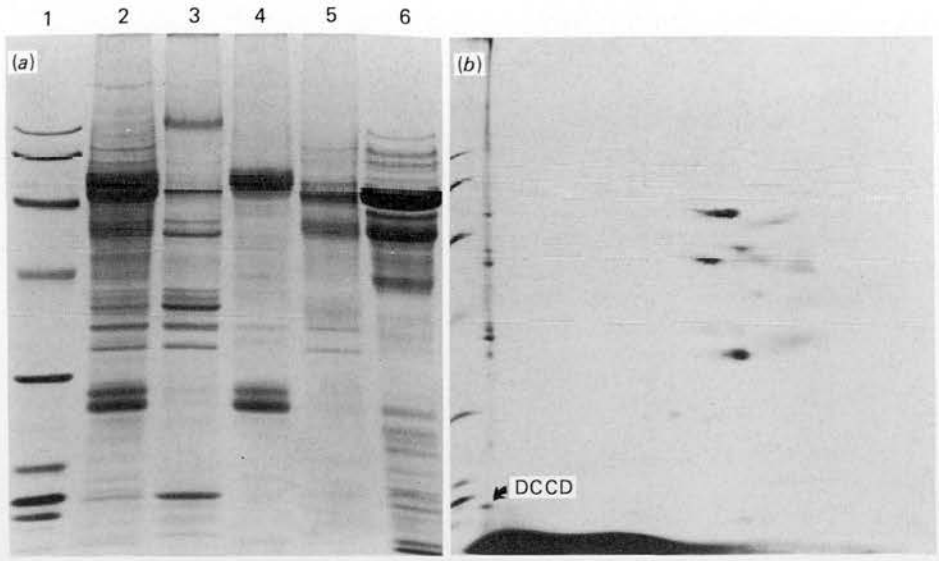



**Fig. 2. Phase separation of chromaffin-granule membrane proteins**

(*a*) Proteins from the Triton X-114 phase-separation procedure were analysed under reducing conditions on an 8–15%-polyacrylamide gel. Track 1, standard proteins, as in Fig. 1. Track 2, complete chromaffin-granule membranes. Track 3, phospholipid-rich phase. Track 4, detergent-rich phase. Track 5, aqueous phase. Track 6, chromaffin-granule lysate (matrix) proteins; the major band is chromogranin A ($M_r$ 70000). (*b*) Two-dimensional analysis of the phospholipid-rich phase. Abbreviation: DCCD, polypeptide that binds [$^{14}$C]dicyclohexylcarbodi-imide (Sutton & Apps, 1981).

as large micellar aggregates formed (Bordier, 1981). A detergent-rich phase was separated from an aqueous phase by low-speed centrifugation through sucrose; it contained about 50% of the membrane protein, leaving about 30–40% in the aqueous supernatant.

Analysis showed that the phospholipid/protein and cholesterol/protein ratios were 3 and 4 times higher, respectively, in the phospholipid-rich phase than in the detergent-rich phase; neither phospholipid nor cholesterol was detected in the aqueous phase. Some 95% of the Triton X-114 was recovered in the detergent-rich phase.

Samples of the three phases were examined by polyacrylamide-gel electrophoresis (Fig. 2*a*). Quite distinct families of proteins are recovered in the three phases, with little overlap between them. This is summarized in Table 1, which gives the distribution of 24 major polypeptides, with $M_r$ values between 104000 and 13000.

## Characterization of chromaffin-granule membrane proteins

In order to characterize the three phases more completely, we analysed each by two-dimensional electrophoresis, identifying proteins where possible by immunological or other methods. We also treated transfers of two-dimensional gels with radioiodinated lectins in order to identify glycoproteins: these are major constituents of chromaffin-granule membranes, but, with a few exceptions, they cannot be observed as Coomassie Blue-stained bands (Abbs & Phillips, 1980; Gavine *et al.*, 1984), so we discuss them separately below.

A two-dimensional gel of the proteins of the phospholipid-rich phase is shown in Fig. 2(*b*). Among these proteins is found a very hydrophobic low-$M_r$ polypeptide that reacts with dicyclohexylcarbodi-imide (Table 1; Sutton & Apps, 1981), which is one component

**Table 1. Distribution of major chromaffin-granule membrane polypeptides**

Identification is based on immune replicas, labelling with [$^{14}$C]dicyclohexylcarbodi-imide (DCCD), and previous work on matrix components (Apps *et al.*, 1985). Key: +, major location; ±, minor location.

| | Phase | | | |
|---|---|---|---|---|
| $10^{-3} \times M_r$ | Phospho-lipid | Deter-gent | Aqueous | Identi-fication |
| 104 | | | + | Matrix |
| 93 | | | + | Matrix |
| 78 | | + | + | Dopamine |
| 75 | | + | | β-hydroxylase |
| 72 | + | | | |
| 70 | | | + | Chromogranin A |
| 68 | | | + | |
| 65 | | + | | |
| 62 | | + | | Matrix |
| 58 | ± | | + | |
| 56 | | | + | Matrix |
| 53 | + | | | |
| 50 | | + | | |
| 47 | + | | | |
| 40 | + | | | |
| 39 | + | | | |
| 37 | + | | | |
| 34 | + | ± | ± | |
| 31 | + | | ± | |
| 26 | | + | | Cytochrome $b_{561}$ |
| 24 | | + | | |
| 17 | + | | | DCCD-reactive |
| 16 | + | | | |
| 13 | + | | | |

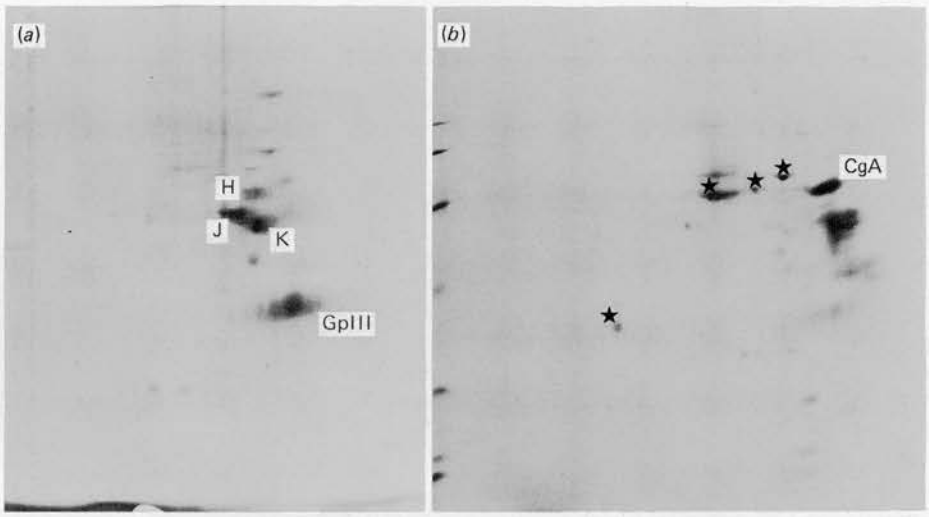



**Fig. 3. Chromaffin-granule membrane proteins in the aqueous phase**

(*a*) Proteins precipitated on removal of detergent; (*b*) proteins remaining in solution after removal of detergent. The proteins labelled in (*a*) are major glycoproteins that stain with Coomassie Blue (cf. Fig. 5); stars in (*b*) denote proteins that are not components of the granule matrix. Abbreviation: CgA, chromogranin A. Standard proteins were as in Fig. 1.

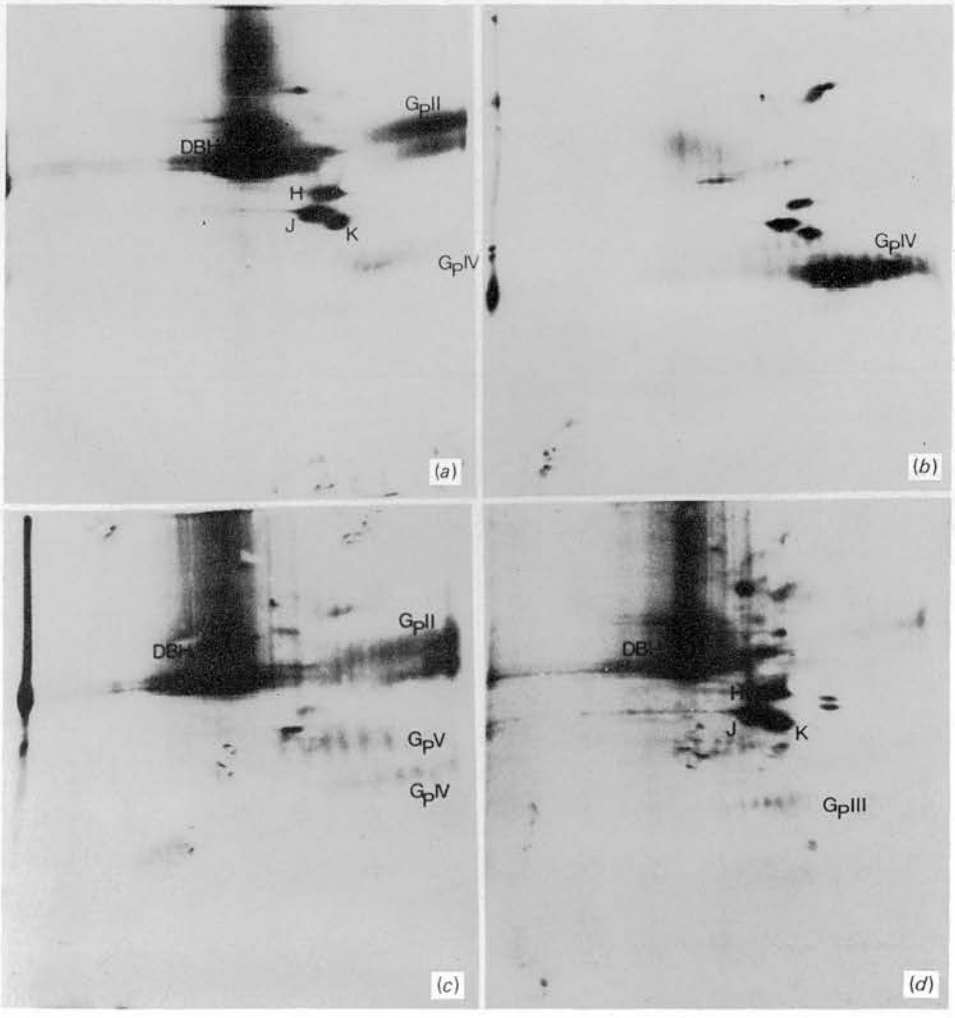



**Fig. 4. Concanavalin A overlays of two-dimensional electrophoretograms of chromaffin-granule membrane proteins**

(*a*) Whole membranes; (*b*) phospholipid-rich phase; (*c*) detergent-rich phase; (*d*) whole aqueous phase. Abbreviation: DBH, dopamine $\beta$-hydroxylase. Labelling of other glycoproteins refers to Fig. 5.

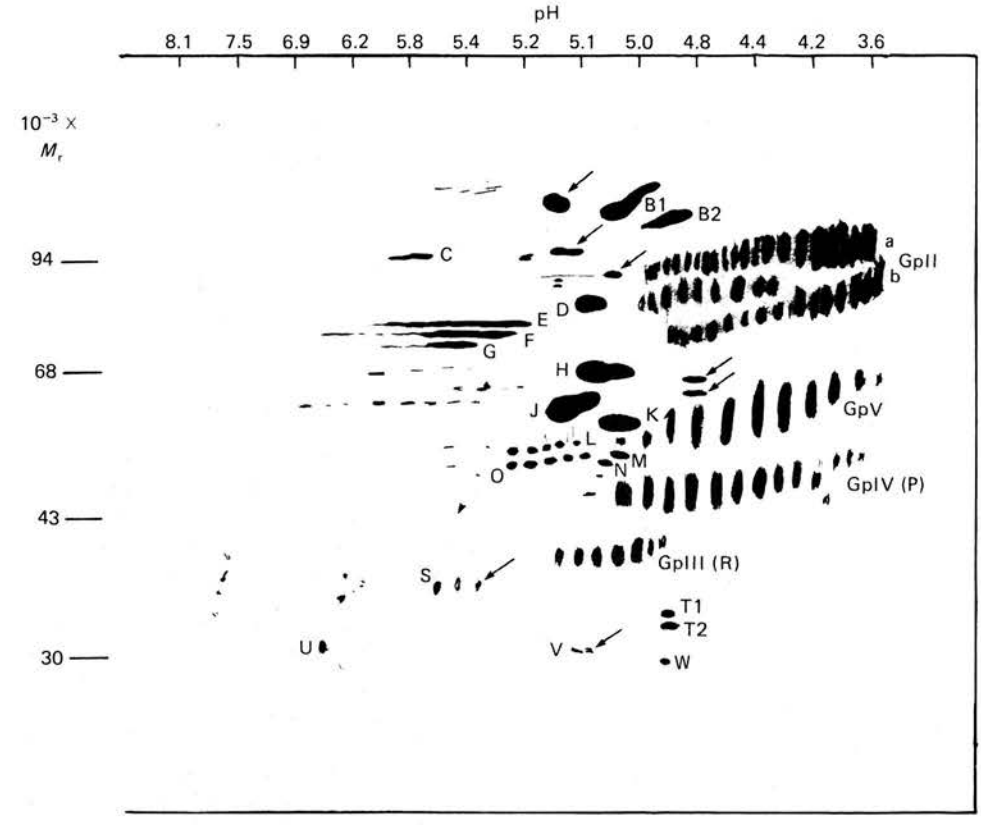



**Fig. 5. Composite drawing of concanavalin A-binding glycoproteins of the chromaffin-granule membrane**

Arrows denote components that remain in solution after dialysis of the aqueous phase. Labelling follows that used by Gavine *et al.* (1984) and Huber *et al.* (1979).

of the proton-translocating ATPase of the membrane (ATPase I). Other components of this enzyme, which we discuss separately below, are also found in this precipitate (Percy *et al.*, 1985), which appears to contain only hydrophobic polypeptides. The high-$M_r$ diffuse band seen in Fig. 2(*a*) is an aggregate: it can be removed by heating the sample before electrophoresis.

The detergent-rich phase is dominated by dopamine $\beta$-hydroxylase and by cytochrome $b_{561}$, the two most abundant proteins of the membrane, which together may comprise over 40% of total protein (Winkler & Westhead, 1980). The cytochrome, which is known to be a trans-membrane integral protein (Abbs & Phillips, 1980; Duong & Fleming, 1984), gives this phase a highly distinctive red colour. The presence of dopamine $\beta$-hydroxylase in the same phase is rather surprising: it is a glycoprotein which is located in both the matrix and the membrane of chromaffin granules. Despite many attempts it has not proved possible to distinguish the two forms convincingly from each other, although Bjerrum *et al.* (1979) suggested that the membrane form could be solubilized by mild proteolysis, and Sabban *et al.* (1983) suggested that the membrane form was a biosynthetic precursor of the soluble form. Others, however, have disagreed with this interpretation (Sokoloff *et al.*, 1985). In our hands this protein (which is unequivocally identified by the immune-replica technique) is found in both the detergent-rich phase and the aqueous phase, consistent with its dual location in granules.

## Chromaffin-granule membrane proteins in the aqueous phase

Two-dimensional gels of the aqueous phase revealed three categories of protein: (1) contaminating matrix proteins; (2) unidentified hydrophilic proteins, possibly arising from the cytosol or from an extracellular location; and (3) several proteins known to be glycosylated, which are thought to be genuine membrane constituents.

The aqueous phase contains a residue (about 0.06%, w/v) of Triton X-114. It was therefore dialysed against Amberlite XAD-2 to remove as much detergent as possible. This resulted in precipitation of 20% of the protein. A two-dimensional electrophoretogram of this material (Fig. 3*a*) revealed several proteins, mostly glycoproteins (see below), previously characterized as membrane components (Gavine *et al.*, 1984). Proteins in categories (1) and (2) remained in solution (Fig. 3*b*) and are essentially the same as those removed from the membranes by $Na_2CO_3$ washing (Fig. 1*b*). Dialysis is thus a simple way of recovering membrane glycoproteins from this fraction.

## Membrane glycoproteins

Because the membrane glycoproteins stain poorly with Coomassie Blue, separated proteins were transferred to nitrocellulose and decorated with three radioiodinated lectins (concanavalin A, wheat-germ agglutinin and lentil lectin) shown previously to bind to the major chro-

**Table 2. Distribution of major chromaffin-granule membrane glycoproteins**

The labelling of components, shown in Fig. 5, follows that of Huber *et al.* (1979) and Gavine *et al.* (1984); GpV has not been named previously. E, F and G are the components of dopamine $\beta$-hydroxylase, as revealed by antibody binding.

| Designation | | Phase | | |
|---|---|---|---|---|
| Gavine *et al.* (1984) | Huber *et al.* (1979) | Phospholipid-rich | Detergent-rich | Pellet from dialysis of aqueous phase |
| $B_1$ | | + | | + |
| $B_2$ | | | + | |
| C | | | + | |
| | GpII | | + | |
| D | | | | + |
| E | | | + | |
| F | GpI | | + | |
| G | | | + | |
| H | | + | | + |
| J | | + | | + |
| K | | + | | + |
| | (GpV) | | + | |
| L | | + | | + |
| M | | | | + |
| N | | | | + |
| O | | | | + |
| P | GpIV | + | | |
| R | GpIII | | | + |
| $T_1$ | | | | + |
| $T_2$ | | | | + |
| U | | | | + |
| W | | | | + |

maffin-granule components. Most of the glycoproteins in fact bind concanavalin A (Gavine *et al.*, 1984), and overlays of the three fractions with this lectin are shown in Fig. 4, together with a composite drawing in Fig. 5. In Fig. 4(*a*) we show an analysis of whole membranes, in order to show the relative intensity of concanavalin A binding of different components before they become concentrated in the separate phases. Figs 4(*b*) and 4(*d*) can be compared with Figs 2(*b*) and 3(*a*), which were stained for protein.

Fig. 4 demonstrates that two major categories of glycoprotein are found; some are revealed as a series of spots spread over a wide range of isoelectric points, whereas others are essentially homogeneous. In at least some cases the former category results from heterogeneous sialylation (Gavine *et al.*, 1984).

The three parts of Fig. 4 demonstrate again that there are clear differences between the three phases, few glycoproteins appearing in more than one (Table 2).

### Application to other subcellular fractions

How applicable is the Triton X-114 method to other membranes? Our knowledge of the component proteins of adrenal-medullary organelles other than secretory granules is only fragmentary, but we have applied the method to a mitochondrial fraction, and to Golgi and rough-endoplasmic-reticulum fractions partially purified from microsomal fractions (Fig. 6). The Golgi-enriched fraction contains some plasma membrane; the enzymic markers for these fractions have been studied (J. G. Pryde & J. H. Phillips, unpublished work).

Fig. 6(*a*) demonstrates that, like chromaffin-granule membranes, the Golgi-enriched microsomal fractions yielded three phases with distinctive polypeptide compositions. In contrast, the less lipid-rich membranes from the mitochondrial and rough-endoplasmic-reticulum preparations (Figs. 6*b* and 6*c*) produced very little initial phospholipid-rich precipitate, although each yielded distinctive detergent and aqueous phases. The method therefore seems to be useful for the initial fractionation of proteins from a variety of types of membrane.

### Distribution of ATPases

Chromaffin-granule membrane preparations are contaminated with traces of mitochondrial $F_1$-ATPase (Apps & Schatz, 1979; Apps *et al.*, 1983; Cidon *et al.*, 1983). The $\alpha$-, $\beta$- and $\gamma$-subunits of this enzyme are revealed by immune replicas in Fig. 7(*a*): these components are found exclusively in the detergent-rich and aqueous fractions. This distribution is also found for the low-$M_r$ dicyclohexylcarbodi-imide-reactive subunit of mitochondrial ATPase (which has an apparent $M_r$ of about 17000; Fig. 7*b*). In contrast, the equivalent chromaffin-granule membrane component (Sutton & Apps, 1981), which is slightly smaller than its mitochondrial counterpart (Fig. 7*b*, track 1), is found solely in the phospholipid-rich phase, where other components of the granule's proton-translocating ATPase are also found (Percy *et al.*, 1985).

Other major dicyclohexylcarbodi-imide-reactive proteins of mitochondria can also be seen in Fig. 7(*b*). That of $M_r$ 50000 is probably the $\beta$-subunit of $F_1$-ATPase (Satre *et al.*, 1982); the band of $M_r$ about 34000 may be the mitochondrial phosphate transporter (Houstek *et al.*, 1981). Fig. 7(*b*), tracks 3, 4 and 5, may be compared with Fig. 6(*c*), in which an equivalent gel stained with Coomassie Blue is presented.

## DISCUSSION

### Separation of chromaffin-granule membrane proteins

The solubilization of chromaffin-granule membranes with Triton X-114, followed by temperature-induced phase separation (Bordier, 1981), has allowed us to separate proteins on the basis of their relative hydrophobicity. The addition of a final detergent-removal step, or the introduction of a preliminary $Na_2CO_3$-wash step, has allowed us to separate the most hydrophilic class, the adherent soluble proteins, and thus to remove proteins that are not true membrane constituents.

The initial precipitate is rich in hydrophobic proteins, typically the dicyclohexylcarbodi-imide-reactive protein and other constituents of ATPase I (Percy *et al.*, 1985). Warming the resultant solution leads to separation of another hydrophobic phase, characterized by its content of dopamine $\beta$-hydroxylase and cytochrome $b_{561}$. The final aqueous medium contains a mixture of soluble proteins and membrane glycoproteins, the latter precipitating on removal of residual detergent. After dialysis we found that the subunits of mitochondrial $F_1$-ATPase (identified by antibody overlays), and residual chromogranins, the major secretory proteins of the adrenal medulla, remained in solution. Dopamine $\beta$-hydroxylase (identified by antibodies and by lectins; Fig. 4*d*) is also found here, arising from its location in the matrix in addition to the membrane. In contrast, the major

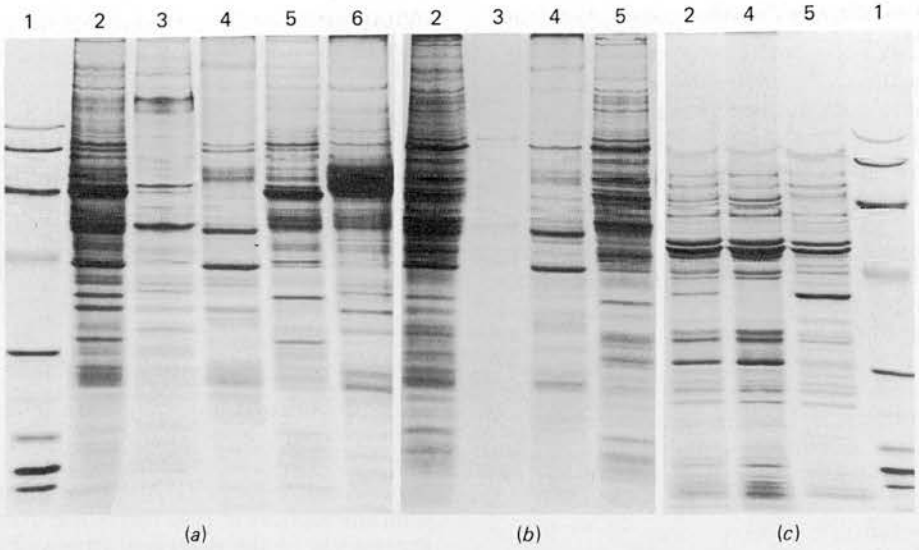



**Fig. 6. Application of phase-separation method to other subcellular fractions**

(*a*) Golgi-enriched microsomal fraction. (*b*) Rough-endoplasmic-reticulum-enriched microsomal fraction. (*c*) Mitochondria. Tracks: (1) standard proteins (see Fig. 1); (2) whole fraction; (3) phospholipid-rich phase (if formed); (4) detergent-rich phase; (5) aqueous phase; (6) chromaffin-granule membranes

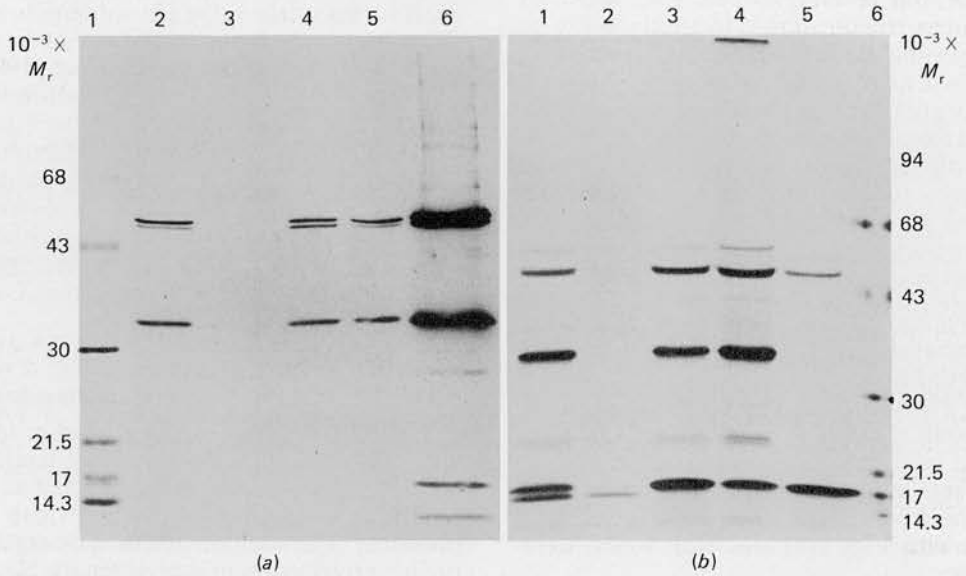



**Fig. 7. Phase distribution of ATPase constituents**

(*a*) Immune replica of electrophoretic separation of chromaffin-granule membrane proteins, obtained by using antiserum to mitochondrial $F_1$-ATPase. Tracks: (1) radioactive standard proteins (as Fig. 1); (2) whole chromaffin-granule membranes; (3) phospholipid-rich phase; (4) detergent-rich phase; (5) aqueous phase; (6) purified mitochondrial $F_1$-ATPase. (*b*) Autoradiograph of one-dimensional separation of proteins labelled by *NN'*-dicyclohexyl[$^{14}$C]carbodi-imide. Tracks: (1) mixture of phospholipid-rich phase from chromaffin-granule membranes with detergent-rich phase from mitochondria; (2) whole chromaffin-granule membranes; (3) whole mitochondrial membranes; (4) detergent-rich phase from mitochondria; (5) aqueous phase from mitochondria; (6) standard proteins.

glycoproteins H, J, K and R (nomenclature of Gavine *et al.*, 1984) were precipitated.

These results therefore do not support the idea that chromogranin A, or any portion of it, may be an integral membrane protein (Settleman *et al.*, 1985). None is revealed by Coomassie Blue staining of the precipitated proteins. The glycoproteins that precipitate are presumably the most hydrophilic of the integral membrane components of the chromaffin granule; many were found to be present to some extent in the phospholipid-rich precipitate as well. They are clearly intrinsic membrane components, even though they do not partition into the detergent phase, as originally suggested by Bordier (1981). A similar partitioning of acetylcholine-receptor pentamers into the aqueous phase has been reported by Maher & Singer (1985).

### Chromaffin-granule membrane glycoproteins

We have previously reported characterizations of the membrane and soluble glycoproteins of chromaffin granules (Gavine *et al.*, 1984; Apps *et al.*, 1985). Much of the complexity of the former analysis has been resolved by the present separation of proteins into three distinct fractions, and the two-dimensional electrophoretograms enable us to observe those components that are highly heterogeneous. Results are summarized in Fig. 5 and Table 2. We have identified the glycoproteins by using the nomenclatures of both Gavine *et al.* (1984) and Huber *et al.* (1979): this is done by comparing binding of the different lectins, as well as by the shapes and positions of spots on the gels.

Early analyses of these glycoproteins concentrated on the major components dopamine $\beta$-hydroxylase, glycoprotein II and glycoprotein III (Fischer-Colbrie *et al.*, 1982). Glycoprotein II appears to be highly heterogeneous ($M_r$ 84000–100000; we have indicated this by the letters a and b in Fig. 5); the wide range of pI presumably results at least in part from incomplete sialylation, since the molecule contains on average 17 $\mu$mol of sialic acid/100 mg of protein (Fischer-Colbrie *et al.*, 1982).

Both glycoprotein II and glycoprotein III have a high affinity for wheat-germ agglutinin: the low affinity of the latter for concanavalin A makes it hard to detect in Fig. 4. Neuraminidase treatment fails to remove all of its heteogeneity, suggesting the possibility of other forms of modification (Gavine *et al.*, 1984).

We believe that glycoprotein IV is identical with the constituent of that name described by Huber *et al.* (1979). A constituent with $M_r$ about 47000, it binds concanavalin A well, and is the most characteristic glycoprotein of the phospholipid-rich phase. It appears to be different from another minor constituent, equally heterogeneous, but mainly found in the detergent-rich phase, that we have called glycoprotein V.

The glycoproteins are summarized in Table 2. We have omitted all glycoproteins that remained in solution after dialysis of the aqueous phase (see Fig. 5), on the assumption that these are either matrix components or extracellular contaminants of the granule preparation. This assumption may not, of course, be valid in every case (Kuchel *et al.*, 1978; Maher & Singer, 1985).

### Generality of the method

The method that we describe in this work appears to be generally useful. Triton X-114 is an effective detergent for solubilizing membrane components, and the simple manipulations of temperature and centrifugation permit an easy first stage of separation of membrane proteins. It is clear that different membranes will behave in characteristic ways. Bordier (1981), for example, found only the detergent-rich and aqueous phases when human erythrocyte membranes or whole myeloma cells were treated, and a similar result was found with platelets (Clemetson *et al.*, 1984) and granulocytes (Pember *et al.*, 1984). We find, however, that relatively lipid-rich membranes yield an additional phase which is insoluble at 0 °C, and which contains its own characteristic family of hydrophobic proteins (Figs. 1 and 6). Furthermore, the two treatments described ($Na_2CO_3$ washing, or dialysis of the aqueous phase) permit a further separation of truly soluble proteins from the most hydrophilic of the intrinsic membrane glycoproteins.

This work was supported by a research grant from the Medical Research Council. We thank Dr. D. K. Apps for his help and for comments on the manuscript, and members of the Cardiovascular Research Unit, University of Edinburgh, for carrying out the phospholipid and cholesterol analyses.

## REFERENCES

Abbs, M. T. & Phillips, J. H. (1980) Biochim. Biophys. Acta **595**, 200–221
Apps, D. K. & Schatz, G. (1979) Eur. J. Biochem. **100**, 411–419
Apps, D. K., Pryde, J. G. & Phillips, J. H. (1980) Neuroscience **5**, 2279–2287
Apps, D. K., Pryde, J. G. & Sutton, R. (1983) Neuroscience **9**, 687–700
Apps, D. K., Phillips, J. H. & Purves, F. (1985) Neuroscience **16**, 477–486
Bartlett, G. R. (1959) J. Biol. Chem. **234**, 466–468
Bjerrum, O. J., Helle, K. B. & Bock, E. (1979) Biochem. J. **181**, 231–237
Bordier, C. (1981) J. Biol. Chem. **256**, 1604–1607
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–256
Cidon, S., Ben-David, H. & Nelson, N. (1983) J. Biol. Chem. **258**, 11684–11688
Clemetson, K. J., Bienz, D., Zahno, M.-L. & Lüscher, E. F. (1984) Biochim. Biophys. Acta **778**, 463–469
Duong, L. T. & Fleming, P. J. (1984) Arch. Biochem. Biophys. **228**, 332–341
Egan, R. W., Jones, M. A. & Lehninger, A. L. (1976) J. Biol. Chem. **251**, 4442–4447
Fischer-Colbrie, R. & Frischenschlager, I. (1985) J. Neurochem. **44**, 1854–1861
Fischer-Colbrie, R., Schachinger, M. Z., Zangerle, R. & Winkler, H. (1982) J. Neurochem. **38**, 725–732
Fisher, R. J., Liang, A. M. & Sundstrom, G. C. (1981) J. Biol. Chem. **256**, 707–715
Garewal, H. S. (1973) Anal. Biochem. **54**, 319–324
Gavine, F. S., Pryde, J. G., Deane, D. L. & Apps, D. K. (1984) J. Neurochem. **43**, 1243–1252
Hartree, E. F. (1972) Anal. Biochem. **48**, 422–427
Higgins, J. A. (1984) Biochem. J. **219**, 261–272
Houstek, J., Pavelka, S., Kopecky, J., Drahota, Z. & Palmieri, F. (1981) FEBS Lett. **130**, 137–140
Howell, K. E. & Palade, G. E. (1982) J. Cell Biol. **92**, 822–832
Huber, E., König, P., Schuler, G., Aberer, W., Plattner, H. & Winkler, H. (1979) J. Neurochem. **32**, 35–47
Kuchel, P. W., Campbell, D. G., Barclay, A. N. & Williams, A. F. (1978) Biochem. J. **169**, 411–417
Maher, P. A. & Singer, S. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 958–962
O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021
Pember, S. O., Heyl, B. L., Kinkade, J. M. & Lambeth, J. D. (1984) J. Biol. Chem. **259**, 10590–10595
Percy, J. M., Pryde, J. G. & Apps, D. K. (1985) Biochem. J. **231**, 557–564
Sabban, E. L., Greene, L. A. & Goldstein, M. (1983) J. Biol. Chem. **258**, 7812–7818
Satre, M., Bof, M., Issartel, J.-P. & Vignais, P. V. (1982) Biochemistry **21**, 4772–4776
Settleman, J., Nolan, J. & Hogue-Angeletti, R. (1985) J. Biol. Chem. **260**, 1641–1644
Sokoloff, R. L., Frigon, R. P. & O'Connor, D. T. (1985) J. Neurochem. **44**, 411–420
Sutton, R. & Apps, D. K. (1981) FEBS Lett. **130**, 103–106
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Winkler, H. & Westhead, E. (1980) Neuroscience **5**, 1803–1823

Received 15 July 1985/4 September 1985; accepted 23 September 1985

# Emerging Technique

# Triton X-114: a detergent that has come in from the cold

James G. Pryde

*The integral proteins of membranes rich in cholesterol and phospholipid can be fractionated by a temperature-induced phase separation in Triton X-114 into three classes differing in their apparent hydrophobicity and free from contamination by adsorbed soluble protein.*

Secretory granule membrane fractions have been purified from many endocrine and exocrine tissues by differential and density gradient centrifugation. Analysis of their proteins by one- and two-dimensional polyacrylamide gel electrophoresis, following solubilization with detergents, has revealed very complex polypeptide compositions. One of the main problems with these preparations is that the membranes are contaminated with adsorbed proteins of extracellular, cytoplasmic and matrix or lumenal origin; this may represent as much as 10% (bovine adrenal secretory granule membranes[1]) or even 30% (rabbit parotid secretory granule membranes[2]) of the total protein. This contamination can be minimized by washing with chaotropic agents, sodium carbonate (high pH) or low concentrations of detergents. However, many of these procedures tend to lead to the loss of biological activities.

Contaminating soluble proteins frequently dominate two-dimensional electrophoretograms of membrane preparations, and since many integral membrane glycoproteins stain poorly with Coomassie blue, a true picture of the relative amounts of integral membrane proteins present may not be obtained. This latter problem has been overcome to some extent by radiolabelling membrane proteins and by probing for glycoproteins with radiolabelled lectins. Differential solubilization studies with non-ionic detergents and the purification of individual integral membrane proteins has been another approach.

The resolving power of electrophoretic techniques would be greatly increased if integral membrane proteins could first be separated into different families, free from contamination by adsorbed soluble proteins. Membrane fractions are normally isolated by differential or isopycnic centrifugation, taking advantage of the size and density of subcellular organelles and the characteristic densities of their membranes; electrophoretic analysis then relies on the charge and size of denatured polypeptides for their separation. Is there some other physical property by which proteins differ that could be used as the basis for a preparative separation technique?

*J.G. Pryde is at the Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK.*



## Phase separation of proteins in Triton X-114

Integral membrane proteins are by definition proteins which have hydrophobic domains that anchor them in the lipid bilayer. Non-ionic detergents with polyoxyethylene head groups can displace and replace much of the normal lipid environment around these proteins; hydrophilic proteins bind very little detergent[3]. The members of the Triton X series of detergents (Fig. 1) form small micelles (140 molecules for Triton X-100) when dispersed in water above their critical micelle concentrations at 0°C. However, at higher temperatures the micelle size increases and, depending upon the number of hydrophilic oxyethylene moieties in the detergent, micellar aggregates form and come out of solution, turning it turbid at a characteristic temperature known as the cloud point. For Triton X-100 (n=9–10), this is at 64°C. For Triton X-114 (n=7–8), however, the cloud point is at the more physiological temperature of 20°C (Refs 3 and 4).

In 1981, Bordier took advantage of both the binding of Triton X-114 to integral membrane proteins, and the formation of a detergent-rich phase when Triton X-114 is warmed to temperatures above its cloud point, to develop a simple and rapid method for the separation of membrane proteins[4]. His idea was to first solubilize a membrane preparation at 0°C, warm it to 30°C, then recover the detergent-rich phase after a low-speed centrifugation. Integral membrane proteins would be recovered from the detergent phase while hydrophilic proteins should remain in the aqueous phase. Bordier tested the efficiency of the phase partitioning with a mixture of hydrophilic proteins containing, among others, serum albumin and cytochrome *c*. He recovered these proteins exclusively from the aqueous phase, which contained very little residual detergent. On the other hand, well-characterized integral membrane proteins such as acetylcholinesterase, bacteriorhodopsin and the subunits of cytochrome *c* oxidase, when solubilized in Triton X-114 at 0°C, partitioned into the detergent-rich phase after warming.

## Solubilization and phase separation of membrane proteins in Triton X-114

The solubilization of integral membrane proteins and phospholipids involves the formation of mixed micelles containing detergent, phospholipid and/or protein. At high ratios of detergent to protein, phospholipid and protein separate into different micelle populations. Triton X-100 is widely used to solubilize membrane proteins and phospholipids and Triton X-114 appears to be equally efficient, at least when it is used at 0°C to solubilize mitochondrial membrane proteins[5]. Bordier used Triton X-114 to solubilize erythrocyte membranes and demonstrated that following a temperature-induced phase separation, about 75% of the integral membrane glycoproteins could be recovered from the detergent phase, while the more hydrophilic proteins associated with the membrane preparation partitioned into the aqueous

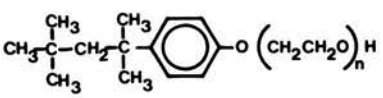


*Fig. 1. Polyoxyethylene p-t-octyl phenol: the Triton X series of detergents. n = average number of ethylene oxide units per molecule, usually greater than 6.*

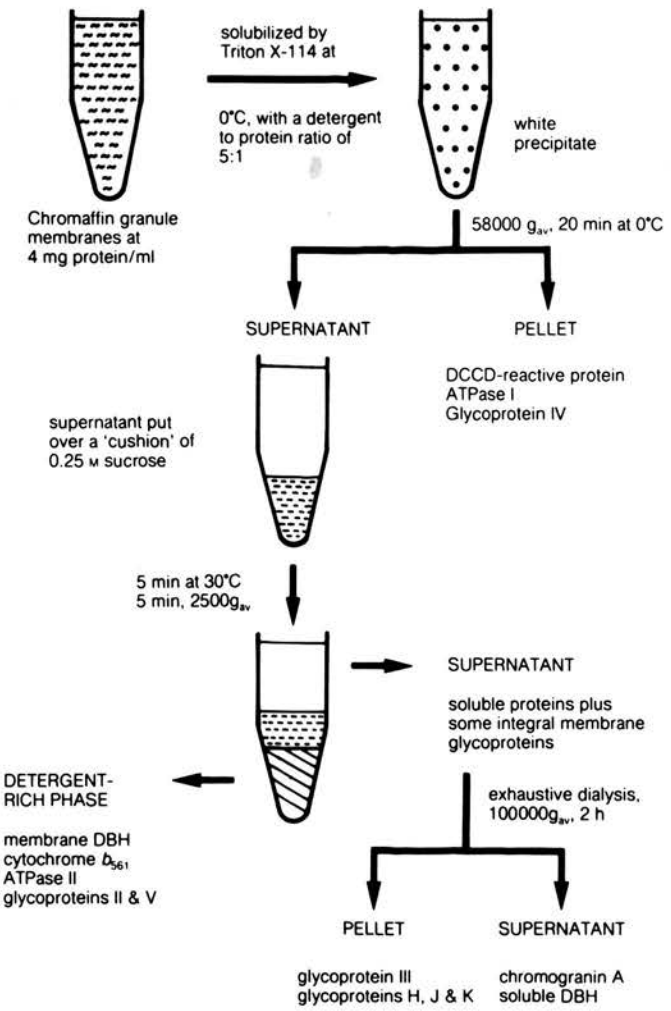



*Fig. 2. Fractionation of chromaffin granule membrane proteins by temperature-induced phase separation in Triton X-114. DHB, dopamine-β-hydroxylase; DCCD, dicyclohexylcarbodiimide.*

phase. A number of recent reports have extended the applicability of this phase partitioning technique to the separation of granulocyte[6] and platelet proteins[7], and to the proteins of the acetylcholine receptor-rich membrane from *Torpedo californica* electroplax[8]. In addition, the phase partitioning properties of Triton X-114 have found a wider application in the separation of cellular and secreted proteins, as Bordier described for mouse myeloma cells[4].

Triton X-114 has been used recently to solubilize the membrane proteins of subcellular fractions from the bovine adrenal medulla and fractionate them by phase partitioning[9]. The catecholamine storage granule (chromaffin granule) membrane has been used as a model to develop a phase separation protocol (Fig. 2) which identifies two families of integral membrane proteins in addition to those integral proteins found, as expected, in the detergent phase; they apparently differ in their degree of hydrophobicity. All are free from contamination by extra-granular and soluble matrix proteins that have complicated two-dimensional electrophoretograms of earlier membrane preparations stained with Coomassie blue[9].

Although rich in cholesterol and phospholipids, chromaffin granule membranes are apparently fully solubilized by treatment with Triton X-114 at 0°C. However, unlike Triton X-100, Triton X-114 fails to maintain all of the membrane components in solution. This can be used to advantage: after about 60 s on ice the solution becomes opalescent, and a precipitate containing about 10% of the total membrane protein and enriched in cholesterol and phospholipid can be recovered by high speed centrifugation. Chromaffin granule phospholipids appear to form large mixed micelles (possibly multilayered bilayer structures) with the detergent, so that aggregation and precipitation occur spontaneously. A subcellular fraction enriched with the marker for the Golgi complex, galactosyltransferase, also produced such a phospholipid-rich precipitate. However, rough endoplasmic reticulum and mitochondria, with their lower ratio of phospholipid to protein, produced no significant precipitate at this stage. One disadvantage with this precipite method is that it has proved difficult to subsequently solubilize the proteins under non-denaturing conditions, for the further purification of ATPase I (see below); this problem is being worked on at present. Analysis of the phospholipid-rich precipitate from chromaffin granule membranes by one-dimensional electrophoresis (Fig. 3a) shows that it contains a unique family of integral membrane polypeptides; a characteristic component is the very hydrophobic dicyclohexylcarbodiimide-reactive protein. All of the subunits of the proton-translocating ATPase (ATPase I) of this membrane are, in fact, confined to this fraction[10]. They are separated from a second ATPase (ATPase II), which is sensitive to vanadate, that is associated with chromaffin granule membrane preparations and partitions into the detergent-rich phase, and also from polypeptides of the mitochondrial $F_1F_0$-ATPase, which contaminate these membrane fractions, and which partition into both the detergent and aqueous phases[9,10].

The major integral membrane proteins of the chromaffin granule remain in solution after solubilization with Triton X-114. When warmed to 30°C, they partition into the detergent-rich phase. This phase is coloured red by cytochrome $b_{561}$, a transmembrane protein[11], and it also contains the amphiphilic form of the enzyme dopamine β-hydroxylase; these two proteins may account for up to 40% of the membrane protein[12]. A soluble form of dopamine β-hydroxylase is present in granule lysates and, as is shown in Fig. 3(a) and 3(b), after phase separation in Triton X-114 small amounts of this enzyme can be found in the aqueous fraction along with soluble secretory proteins. Phase separation in Triton X-114 not only confirms the dual localization of dopamine β-hydroxylase but also shows

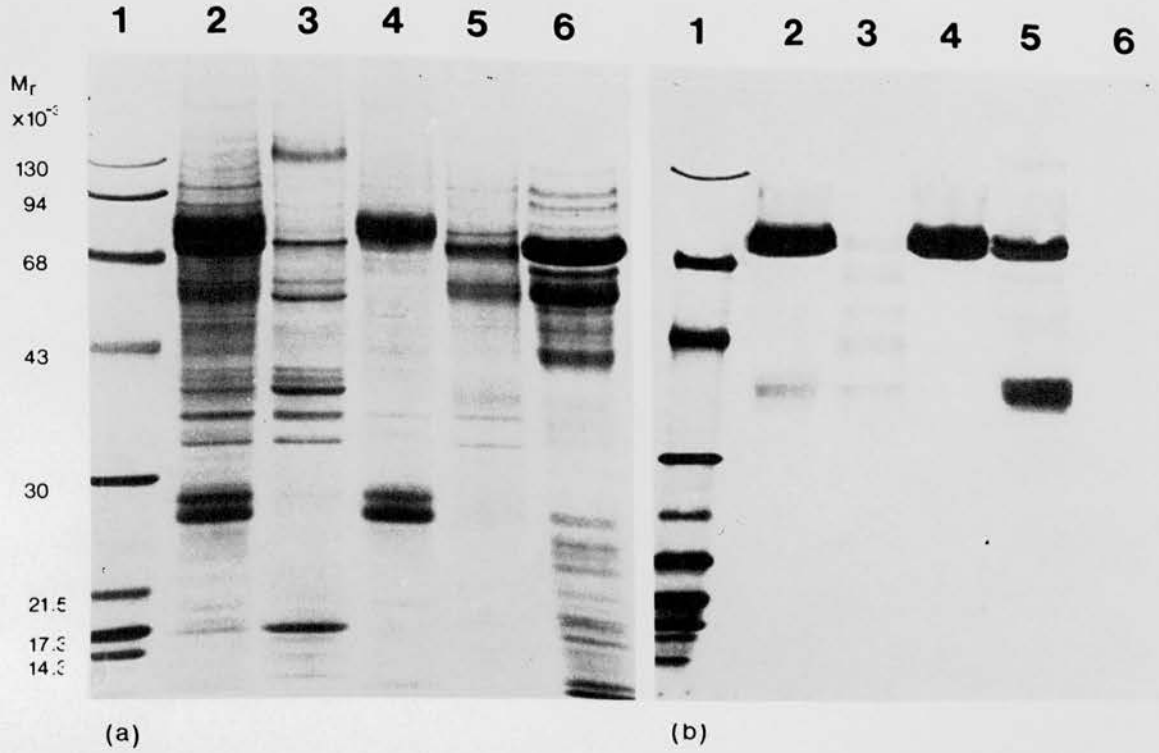



*Fig. 3. Electrophoretic analysis of chromaffin granule membrane proteins after phase separation in Triton X-114. Fractions were analysed in the presence of sodium dodecyl sulphate and β-mercaptoethanol on slab gels containing an 8–15% exponential gradient of polyacrylamide. (a) Electrophoretogram stained with Coomassie blue. Track 1, standard proteins. Track 2, complete chromaffin granule membranes. Track 3, phospholipid-rich phase, showing the subunits of the proton-translocating ATPase, including the very hydrophobic dicyclohexylcarbodiimide-reactive protein ($M_r$ ~ 17 000). Track 4, detergent-rich phase showing dopamine β-hydroxylase ($M_r$ 75 000) and cytochrome $b_{561}$ ($M_r$ 27 000). Track 5, aqueous phase showing chromogranin A ($M_r$ 70 000), the major protein of the chromaffin granule lysate which is shown in Track 6. Track 6, chromaffin granule lysate (matrix proteins). (b) An electrophoretogram identical to that shown in Fig. 3(a) was electrophoretically transferred to a sheet of cellulose nitrate and decorated with [$^{125}$I]lentil lectin. The major lentil binding protein is the membrane form of dopamine β-hydroxylase which partitions into the detergent-rich phase (Track 4). The soluble form of dopamine β-hydroxylase partitions into the aqueous phase (Track 5). The only other major lentil binding protein is glycoprotein III, an integral membrane protein ($M_r$ 37 000) whose anomalous partitioning into the aqueous phase can be seen in Track 5.*

that the two forms can easily be separated by this technique. Labelling studies with impermeant probes have failed to demonstrate that dopamine β-hydroxylase is a transmembrane protein[11,13,14]; however, its partitioning into the detergent-rich phase along with cytochrome $b_{561}$ suggests that this large tetrameric glycoprotein may contain a hydrophobic domain responsible for anchoring it to the inner (matrix) face of the chromaffin granule membrane.

**Integral membrane glycoproteins in the aqueous phase**

As the number of membrane preparations subjected to phase-partitioning in Triton X-114 has increased, an anomalous partitioning of integral membrane glycoproteins into the aqueous phase has been observed[7–9]. Figure 3(b) shows an autoradiogram of the fractionated chromaffin granule membrane proteins shown in Fig. 3(a) probed with radiolabelled lentil lectin. The partitioning of a major chromaffin granule glycoprotein, known as glycoprotein III, into the aqueous phase can be seen. Three other integral membrane glycoproteins which bind concanavalin A can also be detected in this fraction[9]. The partitioning of these membrane glycoproteins into the aqueous phase suggests that while they may bind detergent they appear to be excluded from detergent micelles. The aqueous phase does contain residual Triton X-114 which, at about 700 μM, is three or four times more concentrated than its critical micelle concentration[4]. Of the many possibilities which may explain this anomalous partitioning behaviour, two may be considered and tested experimentally.

(1) The large hydrophilic moieties on these glycoproteins prevents their hydrophobic membrane domains from being intercalated into the hydrophobic interior of the detergent micelle without disrupting the micellar structure. Thus, both the acetylcholine receptor and glycoprotein III are acidic glycoproteins which show positive periodate–Schiff staining, indicative of a significant carbohydrate content[15,16]. The non-covalent association of integral membrane proteins with peripheral proteins, such as those from the cytoskeleton, may also predispose such complexes to partition into the aqueous phase.

(2) Some membrane glycoproteins, which require detergent for their initial solubilization, subsequently associate in solution to form oligomers which remove their hydrophobic domains from the surrounding hydrophilic environment. Such behaviour has been observed for a number of integral membrane proteins[17], such as the Thy-I antigen on lymphocytes[18] and for cytochrome $b_5$[19].

To test whether the chromaffin

granule membrane glycoproteins partitioned into the aqueous phase as a result of their hydrophobic membrane domains remaining masked by bound detergent, exhaustive dialysis was carried out to remove the residual Triton X-114 from the aqueous phase. As Triton X-114 has a low critical micelle concentration and consequently has a low monomer concentration in solution, a hydrophobic resin (Amberlite XAD-2) was placed in the dialysis buffer to facilitate removal of the detergent. By removing the detergent one would expect integral membrane proteins to become insoluble and to aggregate[20], while truly soluble proteins and those exceptions which, like Thy-I (Ref. 18), form soluble complexes, should remain in solution.

After several days of dialysis about 90% of the residual detergent was removed from the aqueous phase obtained from the partitioning of chromaffin granule membranes, and a precipitate was recovered which contained about 20% of the sample protein. Two-dimensional electrophoresis of the proteins in this precipitate identified four integral membrane glycoproteins, including glycoprotein III, while secretory proteins like chromogranin A and the soluble form of dopamine β-hydroxylase remained in solution[9]. This simple expedient of dialysing the aqueous phase suggests that it was bound detergent keeping these chromaffin granule membrane glycoproteins in solution and it allowed the separation of this particular family of integral membrane proteins.

Chromogranin A is the major secretory protein discharged from chromaffin cells along with catecholamines and opioid peptides. It has been claimed recently that chromogranin A is an integral membrane protein[21]. However, Chromogranin A and other adherent soluble proteins can be progressively removed from the chromaffin granule membrane by washing with sodium carbonate, and two-dimensional maps of polypeptides washed from the membranes are identical to maps of the granule matrix proteins apart from an additional one or two polypeptides of extragranular origin[9]. The fact that chromogranin A remains in solution after phase separation in Triton X-114 and subsequent dialysis, strongly suggests that it is not an integral membrane protein[1,9].

## Conclusions

Temperature-induced phase separation of Triton X-114-solubilized membrane proteins readily separates such proteins into two families. In favourable cases a third, apparently very hydrophobic category is separated; and a final dialysis step separates a fourth category (Fig. 2). Within a few hours these fractions can be isolated and put through appropriate washing procedures. The simplicity of these manipulations, the reproducibility of the polypeptide patterns obtained, and the retention of biological activity, make this a potentially valuable technique for membrane research. Separations can be carried out on the microscale or on a large preparative, and possibly even an industrial, scale.

Triton X-114 has become an invaluable tool for the dissection of the chromaffin granule. Solubilization of its phospholipid-rich membrane produces a precipitate containing a unique family of hydrophobic proteins in addition to those of the detergent phase. The integral membrane proteins which partition anomalously into the aqueous phase can be recovered following dialysis. These procedures open up many new avenues of investigation; for example, the phase separation of dopamine β-hydroxylase will allow comparison of the membrane form of this enzyme with its soluble counterpart and provide the basis of an assay for studying the role played by its hydrophobic domain in its association with the membrane. Comparative electrophoretic studies with other secretory systems should now be possible, especially since contaminating secretory proteins can be removed and families of integral membrane proteins compared.

## Acknowledgements

This work was supported by a research grant from the Medical Research Council. I thank Dr David K. Apps and Dr John H. Phillips for their comments on the manuscript.

## References

1 Winkler, H. (1976) *Neuroscience* 1, 65–80
2 Castle, J.D. and Palade, G.E. (1978) *J. Cell Biol.* 76, 323–340
3 Helenius, A. and Simons, K. (1975) *Biochim. Biophys. Acta* 415, 29–79
4 Bordier, C. (1981) *J. Biol. Chem.* 256, 1604–1607
5 Egan, R.W., Jones, M.A. and Lehninger, A.L. (1976) *J. Biol. Chem.* 251, 4442–4447
6 Pember, S.O., Heyl, B.L., Kinkade, J.M. and Lambeth, J.D. (1984) *J. Biol. Chem.* 259, 10590–10595
7 Clemetson, K.J., Bienz, D., Zahno, M-L. and Luscher, E.F. (1984) *Biochim. Biophys. Acta* 778, 463–469
8 Maher, P.A. and Singer, S.J. (1985) *Proc. Natl Acad. Sci USA* 82, 958–962
9 Pryde, J.G. and Phillips, J.H. (1986) *Biochem. J.* 233, 525–533
10 Percy, J.M., Pryde, J.G. and Apps, D.K. (1985) *Biochem. J.* 231, 557–564
11 Duong, L.T. and Fleming, P.J. (1984) *Arch. Biochem. Biophys.* 228, 332–341
12 Winkler, H. and Westhead, E. (1980) *Neuroscience* 5, 1803–1823
13 Huber, E., Konig, P., Schuler, G., Aberer, W., Plattner, H. and Winkler, H. (1979) *J. Neurochem.* 32, 35–47
14 Abbs, M.T. and Phillips, J.H. (1980) *Biochim. Biophys. Acta* 595, 200–221
15 Karlin, A., Weill, C.L., McNamee, M.G. and Valderrama, R. (1975) *Cold Spring Harbor Symp. Quant. Biol.* 40, 203–210
16 Fischer-Colbrie, R., Schachinger, M.Z., Zangerle, R. and Winkler, H. (1982) *J. Neurochem.* 38, 725–732
17 Tanford, C. and Reynolds, J. (1976) *Biochem. Biophys. Acta* 457, 133–170
18 Kuchel, P.W., Campbell, D.G., Barclay, A.N. and Williams, A.F. (1978) *Biochem. J.* 169, 411–417
19 Spatz, L. and Strittmatter, P. (1971) *Proc. Natl Acad. Sci. USA* 68, 1042–1046
20 Singer, S.J. (1974) *Annu. Rev. Biochem.* 3, 805–833
21 Settleman, J., Nolan, J. and Hogue-Angeletti, R. (1985) *J. Biol. Chem.* 260, 1641–1644